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IN VITRO EFFECT OF ADRENAL CORTICAL EXTRACT UPON LYMPHOCYTOLYSIS

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INTRODUCTION

LITTLE is known concerning the mechanism whereby adrenocortical hormones (ACH) produce lymphocyte dissolution *in vivo* (White and Dougherty, 1946). There is evidence, however, that adrenocortical steroids do not act directly upon lymphocytes to produce lysis. Thus, if has been reported that whole adrenal cortex extract (ACE) does not produce breakdown of the lymphocytes present in blood following *in vitro* incubation (Hechter, 1948) or of lymphocytes, obtained from lymph nodes, incubated *in vitro* in diluted homologous serum (Robertson, 1948). On the other hand, there is suggestive evidence that adrenocortical steroids accelerate *in vitro* lymphocyte breakdown provided that lymphoid tissue is present in the system. Thus, indirect evidence for increased lymphocyte dissolution has been obtained: (a) following addition of ACE, but not desoxycorticosterone acetate, to blood perfused through the isolated rabbit spleen (Hechter, 1948), and (b) in tissue culture experiments wherein 11-dehydrocorticosterone and 11-dehydro-17-hydroxycorticosterone increased the rate of degeneration of small and medium sized lymphocytes in the migration zone of lymph node implants (Heilman, 1945).

These *in vitro* studies taken together with the evidence that lymphoid tissue is the predominant site of lymphocyte breakdown *in situ* (White and Dougherty, 1946, Ehrich, 1946), raise the possibility that the *in vivo* lymphocytolytic activity of ACH requires a factor present in lymphoid tissue. Studies were therefore undertaken to investigate the *in vitro* breakdown of lymphocytes in the presence of homogenates of lymphoid tissues and other organs and in other media both in the presence and absence of added steroids.

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METHODS

General: Lymphocyte suspensions obtained from the pooled lymphoid organs of rabbits were incubated with either tissue homogenates, homologous defibrinated blood or serum, or buffered salt solutions in the presence and absence of either ACE or crystalline steroids, using a clean but non-aseptic technic. Lymphocyte breakdown was evaluated by counting cells before and after various periods of incubation, usually one hour. To permit rapid differentiation of lymphocytes from spherical fat globules, and "debris" present in tissue homogenates, the cells were stained with brilliant-cresyl blue and counted under high dry magnification. Evidence will be presented later in this section of the paper that satisfactory cell counts are obtained with this procedure only under strictly defined conditions. Lymphocyte breakdown, as measured with this technic, thus refers to the disappearance of cells from the medium. Since the stainable cell structure observed is almost entirely nuclear, rupture of the cytoplasmic wall, unless simultaneously accompanied by nuclear dissolution, would not be measureable as lymphocytolysis. The evaluation of lymphocyte breakdown by a counting method requires that representative samples for cell counts be obtained from the incubation medium; thus, two separate counts were generally made to determine cell concentration and the sample was examined for cell agglutination or absorption upon particulate matter present in homogenates. These complications, however, were encountered but infrequently, and it would therefore appear that the lymphocyte disappearance observed was predominately due to lymphocyte breakdown.

It should be emphasized that the lymphocyte suspensions in the various experiments contain variable numbers of cells which are non-viable in that they are freely permeable to 1:1,000 eosin in Tyrode, in contrast to viable lymphocytes which are eosin-resistant (cf. Schrek, 1946a and 1946b); and finally, that the different suspensions contained variable percentages of lymphocytes derived from spleen, thymus and lymph nodes.

Experimental Animals: Rabbits, 3–5 months old, of various strains, weighing between 2.5 and 4.0 kg. were used. Marked variations were observed from animal to animal with respect to the individual weights of thymus, lymph nodes and spleen and in the total number of lymphocytes obtainable from the combined lymphoid organs studied. The latter variations could not be correlated to the weight of pooled lymphoid tissues or to the body weight of the rabbit.

Lymphocyte Suspensions: After the animal was killed by exsanguination, the spleen, thymus and cervical lymph nodes were removed, trimmed of fat and connective tissue, and weighed. The organs were minced with scissors in the presence of 10 to 15 cc. of either 0.9 per cent NaCl (hereafter referred to as saline) or Tyrode solution (adjusted to pH 7.4) and filtered through 4–6 layers of muslin. Excluding erythrocytes, practically all of the cells in these suspensions are lymphocytes and of these 75–85 per cent were small, 6–8 per cent large, and the remainder were classed as medium-sized as determined on air-dried smears stained with Wright's stain. The suspensions were prepared in the cold using chilled solutions and stored in the refrigerator until the experiment was initiated (usually 1–2 hours later). Those cell suspensions which were not treated further are contaminated with lymphoid tissue components, and will hereafter be referred to as "non-washed" cells. In ex-

periments where it was desired to remove the bulk of these contaminants the cells were separated by slow speed centrifugation (15 minutes at 1,000 RPM) in a cold room; the supernatent was discarded and replaced with fresh salt solution. A small amount of stringy material which does not go into the suspension was removed by filtration through muslin. Such cell suspensions even though they may be contaminated to a degree will be referred to as "washed" lymphocytes.

In other experiments, lymphocyte suspensions were prepared by direct homogenization of the lymphoid tissues in isotonic or near isotonic salt solutions using a *"worn"* Potter and Elvejhem (1936) homogenizer. The lymphoid tissue was directly homogenized with saline or more frequently with a solution containing equal parts of saline-phosphate buffer (pH 7.00 or 7.38) using 1.5 cc. solution per gram of tissue. The lymphocytes in these preparations (after staining air-dried smears with Wright's stain) are similar in appearance to stained preparations obtained by saline or Tyrode extraction of minced lymphoid tissue. However, these cells, in contrast to those obtained with saline, are uniformly freely permeable to eosin and thus may be assumed to be "non-viable." The lymphocytes in such homogenized suspensions will therefore be designated as "damaged" lymphocytes.

Homogenates: The lymphoid residue, remaining on the muslin after the lymphocyte suspension had been filtered, furnished the material for the lymphoid homogenate. The homogenates were prepared in the following manner: 1 part of tissue residue was homogenized with 1.5 parts of either iced distilled water or a solution consisting of equal parts of 1 per cent sodium chloride and M/15 phosphate buffer (pH 7.0) in the apparatus of Potter and Elvejhem. The homogenizing tube was held in ice water during the course of homogenization, and grinding was performed at intermittent intervals of 30–45 seconds. Upon completion of homogenization, the material was filtered through cheese cloth and 0.5 cc M/15 phosphate buffer (pH 7.0) was added to every 1 cc. of the water homogenate. Where homogenates were prepared with salt-phosphate solution instead of water, no further additions were made.

The number of cells in these lymphoid homogenates relative to the concentration of lymphocytes used in any experiment was so small (less than 3 per cent) that for practical purposes, these homogenates were regarded as "cell free."

Homogenates of brain and muscle were prepared similarly, except that these tissues were not extracted with salt solutions prior to homogenization. Homogenates of brain and muscle contain only a few cells which stain with cresyl-blue.

Steroids Investigated: The ACE utilized in these studies was a dried neutral ethyl acetate residue of hog adrenals furnished by Dr. M. Kuizenga of the Upjohn Company. The preparation contained 1.06 Ingle Work Units per mg. In addition to the ACE preparation, the following crystalline steroids were studied: corticosterone, 11-dehydrocorticosterone (Compound A of Kendall), 11- dehydro-17 hydroxycorticosterone (Compound E of Kendall), 11- desoxycorticosterone, 11-desoxycorticosterone acetate, α estradiol, ethyl testosterone, 17-hydroxyprogesterone, androsterone and cholesterol. Solutions of these steroids and ACE were prepared using redistilled acetone as solvent. Aliquot portions were added to test tubes (14×85 mm.) in which

the lymphocyte incubation studies were subsequently to be performed; the solvent was removed *in vacuo* and the tubes containing dried steroid were stored at 2° C. until used for experiments.

Evaluation of Lymphocyte Breakdown: Lymphocyte suspensions were pipetted into the tubes containing the dried steroids, and then an equal volume of either serum, blood, saline (buffered to pH 7.0 with M/150 phosphate or borate) or tissue homogenate added. (Where the "damaged" lymphocytes containing homogenized lymphoid tissues were tested, only the single suspension was employed.) The tubes were stoppered, mixed and samples were withdrawn to determine the initial cell concentration. Tubes were inserted in a machine which shook them horizontally 230 times a minute at 38°C. To insure thorough mixing a glass rod was added to each tube. The experiments were so designed that two tubes, containing no steroid, were controls for ACE or the other steroids investigated.

Samples from the incubation tubes were removed with either red or white blood cell pipettes, and a solution of 0.172 per cent brilliant cresyl-blue (C.I. No. 877, Nat. Aniline Div.) containing 0.04 per cent sodium cyanide was used as a diluting fluid. Cresyl-blue cyanide stains leucocytes blue, and hemolyzes erythrocytes. The cells were diluted either 1:10, 1:20, 1:100 or 1:200, so that at least 200 cells could be counted on 4 mm.^{2} of the hemocytometer chamber. The pipette was gently shaken with a rotary motion for 3 minutes in a standardized manner. Only a single counting chamber was used in these experiments, and all pipetting and counting of cells were made by a single observer using a code system, so that each sample was an unknown.

Comparative studies using brilliant cresyl-blue cyanide and 1 per cent acetic acid as diluents on whole heparinized rabbit blood revealed that the total leucocyte count, with both diluents was almost identical. It was further demonstrated (using phosphate buffers) that variations of pH between 6.0 and 8.0 in the samples did not affect the capacity of the brilliant cresyl-blue cyanide to stain lymphocytes. Shifts in the pH of the incubation medium containing lymphocytes, therefore, could not give rise to false results on the basis that lymphocytes failed to take the stain at particular pH values.

Early in the course of this work it was discovered that significant lysis of of the cresyl-blue stained cells may occur in the blood pipette on standing at room temperature, and that the degree of this lysis was a function both of temperature and time. The error introduced as the result of this lysis, however, could be removed by the following procedure which was therefore uniformly employed: The incubated tubes were removed from the incubator and immediately chilled in an ice bath. Samples were obtained from these iced tubes after mixing; the cells were then diluted with cold cresyl-blue and the blood cell pipette containing the diluted suspension was then placed in the refrigerator at 2° until the cell count was actually made. Where lymphocyte breakdown was followed over a course of several hours, the tubes containing the incubated cells were not chilled, otherwise the remainder of the procedure described above was followed. The duplicability of counts taken under the above conditions is shown by the fact that when counts were made on two samples containing the identical constituents, in 100 consecutive cases the average percentage differences between the counts was 3.0 per cent ($\pm$0.40, standard error) despite the fact that one sample was counted 30–40

minutes after the other. This difference, however, is not the "error" of a single count; from the studies of Berkson et al. (1940) who have evaluated the errors involved in cell counts with a hemocytometer, it may be calculated that a single count, where 200 cells are counted, can be determined significantly only within about ±20 per cent.

Counts on Eosin-Resistant and Eosin-Stainable Cells: To obtain information concerning the percentage of "eosin-resistant" and "eosin-stainable" cells in a given sample, separate counts were made using brilliant cresyl-blue and eosin 1:1000 in Tyrode (pH 7.4) as diluents. The total lymphocyte concentration was obtained from the count of cresyl-blue stained cells; the eosin-stained cells were next counted directly and the eosin-resistant cells were obtained by difference.

Lymphocytolysis in the Absence of Added Steroids

Before starting to examine the effect of ACE or other steroids, studies were undertaken to evaluate lymphocyte breakdown in the absence of added steroids. A summary of these studies, as they pertain to the subject of this communication, will be presented; full details will be reported elsewhere.

Lymphocyte suspensions undergo spontaneous breakdown upon storage in Tyrode or saline solution and upon incubation at 38° in serum or tissue homogenates. Freshly prepared cell suspensions are most stable in the cold (2° C.) and at slightly acidic pH's; elevation of temperature to 25° or 38° and pH to 7.4 increases the rate of spontaneous breakdown of cells. The rate of lymphocytolysis of cell suspensions stored in the cold is independent of the initial cell concentration (range 377,000 to 82,000 lymphocytes per $mm.^3$) and is not directly related to the percentage of eosin-stainable cells initially present in the suspension. It appears likely that eosin-resistant cells are transformed to eosin-stainable cells prior to dissolution, and that the percentage of non-viable eosin-stainable cells in a suspension at a particular time is the resultant of the rates of conversion of eosin-resistant to eosin-stainable cells and the dissolution of the latter cells.

Upon incubating "washed" and "non-washed" lymphocyte suspensions in defibrinated blood, serum or tissue homogenates at 38° C., lymphocytolysis proceeds at a significant rate in the absence of steroids; consequently any effects of ACE or other steroids to be described later is to *alter the rate of lymphocytolysis and not to initiate this process.* It was uniformly observed that the degree of "spontaneous" lymphocytolysis during incubation was greatest with lymphoid homogenate and least with serum; lymphocyte breakdown in brain and muscle homogenates was somewhat greater than in serum but did not approach the rate observed with lymphoid homogenates. Lymphocytolysis of cells added to defibrinated blood is approximately equivalent to that observed in serum. Lymphoid homogenates, in addition to increasing the rate of lymphocytolysis, exhibit other evidences of cytotoxicity. Thus, lymphocytes incubated in lymphoid

homogenates noticeably decrease in cell volume after one hour incubation, and after 2.5 hours, in some cases, the cell size has diminished to such an extent that it is not possible to count the cells accurately using our usual magnification (×400). On the other hand, those lymphocytes incubated for 3 hours in Tyrode, whole blood, serum, or brain and muscle homogenates which have not undergone dissolution, retain their initial size or, if anything, tend to swell. In addition, lymphoid homogenates rapidly increase the permeability of lymphocytes to eosin so that soon after incubation has been initiated almost all of the lymphocytes present in the medium are "eosin-stainable". This effect upon the cytoplasmic permeability of lymphocytes is more or less specific for lymphoid homogenates and is not observed with Tyrode, serum or whole blood and only occurs to a limited extent with homogenates of brain and muscle.

The process of washing lymphocytes (*i.e.* centrifugation, etc.) does not significantly influence the stability or permeability of lymphocytes. However, the so-called "damaged" lymphocytes are more labile than the "washed" or "non-washed" lymphocyte suspensions and breakdown more rapidly (about 50%) than either "washed" or "non-washed" lymphocytes in lymphoid homogenates.

Considerable variation in the degree of lymphocyte breakdown was observed upon incubating cells in serum or tissue homogenates in the individual experiments under apparently identical conditions. The variations encountered may be due to the fact that (a) the lymphocyte suspensions were obtained from rabbits, heterogenous in age, weight and strain; (b) the cell suspensions were stored, in the cold, for various periods of time; and (c) the media employed for incubation were obtained from different animals. Of these factors (a) seems the most important since the marked variations persisted when lymphocytes from different animals were studied in a single medium (serum) under identical conditions.

Lymphocytolytic Index

In preliminary testing, it was noted that while ACE almost regularly increased the rate of cytolysis of cells incubated in lymphoid homogenates relative to the non-steroid control, the differences produced by ACE were small relative to the marked variation in the rate of lymphocytolysis in the non-steroid controls. Accordingly a lymphocytolytic index was devised, which is independent of the rate of lymphocyte breakdown in the control. For this purpose, the difference in cell count after incubation (one hour) between the treated sample and the non-steroid control ("Δ") was determined. From this Δ, the difference due to treatment per 100 cells of the control sample (henceforth referred to as Δ%) was calculated. For example, if after one hour of incubation the cell concentration per mm.^{3} is 200×500 in the control and 160×500 in the ACE-treated sample the dif-

ference due to ACE at one hour (ΔACE) is -40×500 and the Δ% is $\frac{-40\times500}{200\times500}\times100$ or -20. Using the Δ% index, the non-steroid ACE control is automatically zero and increased cell breakdown is expressed as a negative Δ% value, while inhibition of lymphocytolysis yields positive values. To ascertain the statistical significance of Δ% values obtained in the presence of hormonal steroids, it was necessary to have information concerning the error of the method. This was determined by evaluating the Δ% value of an inert steroid, cholesterol,

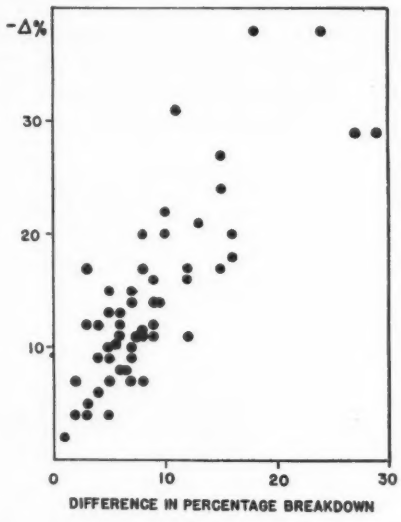

FIG. 1 illustrates the relationship between Δ% and the difference in percentage breakdown induced by ACE (10 μg. per cc.) upon varying types of lymphocytes incubated in lymphoid homogenate for one hour at 38° C. Each dot represents an individual experiment.

arbitrarily tested at a concentration of 10 μg. per cc. under varying conditions. Under all conditions it was observed that Δ% values obtained with cholesterol are not significantly different from zero, as evaluated by the null method. Consequently the significance of Δ % values obtained with ACE or other steroids was determined by comparing the results obtained with those of the appropriate cholesterol group using the "t" test of Fisher (1936).

One may also evaluate lymphocyte breakdown by determining the difference in percentage lymphocyte breakdown in a given interval in the absence and presence of a steroid. However, in contrast to the Δ% index, the values obtained with this procedure are influenced by the degree of spontaneous lymphocyte breakdown and tend to be

low when the latter factor is high. Figure 1 illustrates the relationship between Δ% ACE and the difference in percentage breakdown produced by ACE in experiments where lymphocytes were incubated in lymphoid homogenates for one hour. It will be seen, in general, that Δ% ACE is proportional to the difference in percentage breakdown produced by ACE; those points which deviate from the linear curve at high Δ% values are experiments wherein a high degree of spontaneous breakdown was obtained.

Effect of ACE

In preliminary experiments, where ACE was incubated at 38° with lymphocytes in the presence of lymphoid homogenates for several hours, the lymphocytolytic activity of ACE tended to be most pronounced at the first hour of incubation. This finding, combined with the previously described observation of lymphocyte shrink-

TABLE 1. THE EFFECT OF ACE CONCENTRATION UPON THE BREAKDOWN OF "NON-WASHED" LYMPHOCYTES INCUBATED WITH LYMPHOID HOMOGENATES AT 38° FOR ONE HOUR

Steroid	Concentration	Number experiments	Mean Δ%	p†
	μg. per cc.			
ACE	1	5	− 5.2±1.8*	0.02–0.05
	5	5	− 8.2±2.1	<0.01
	10	5	−13.0±1.8	<0.01
	50	5	−13.4±2.2	<0.01
	100	5	− 8.4±2.0	<0.01
Cholesterol	10	8	− 1.5±1.3	—

* Standard error of the mean.
† P value as compared to the cholesterol mean; when "p" is less than 0.05, the difference is regarded as statistically significant.

age in lymphoid homogenates, led us to evaluate the lymphocytolytic activity of ACE (and other steroids studied) after an arbitrary period of one hour of incubation at 38° C.

Effect of ACE Upon Cells Incubated in Lymphoid Homogenate: In Table I data are presented illustrating the effect of ACE concentration upon lymphocytes incubated with homogenates of lymphoid tissue for 1 hour. In each of these 5 experiments "non-washed" lymphocytes from a single suspension were incubated with equal volumes of a single homogenate for one hour and the ACE concentration was varied from 1 to 100 μg per cc. To assess the significance of the ACE effects, the collected data of other experiments where cholesterol (10 μg. per cc.) was tested under similar conditions (*i.e.* "non-washed" lymphocytes incubated in lymphoid homogenates) is also shown in Table I. It will be seen that maximal lymphocytolytic activity as evaluated by the Δ% index is obtained with the 10 μg. cc. dose. The significance of the drop in lymphocytolytic activity

noted when the ACE concentration is increased from 10 to 100 μg. per cc. remains to be determined; it should, however, be noted that the difference in response between the 10 and 100 μg. per cc. doses of ACE is not significant, in a statistical sense, and that cholesterol was not tested at a level of 100 μg. per cc.

Figure 2 shows a comparison of the lymphocytolytic activity of ACE upon "washed", "non-washed" and "damaged" suspensions incubated in the presence of lymphoid homogenate. In these experiments a 10 μg. per cc. ACE dosage was employed, and cholesterol

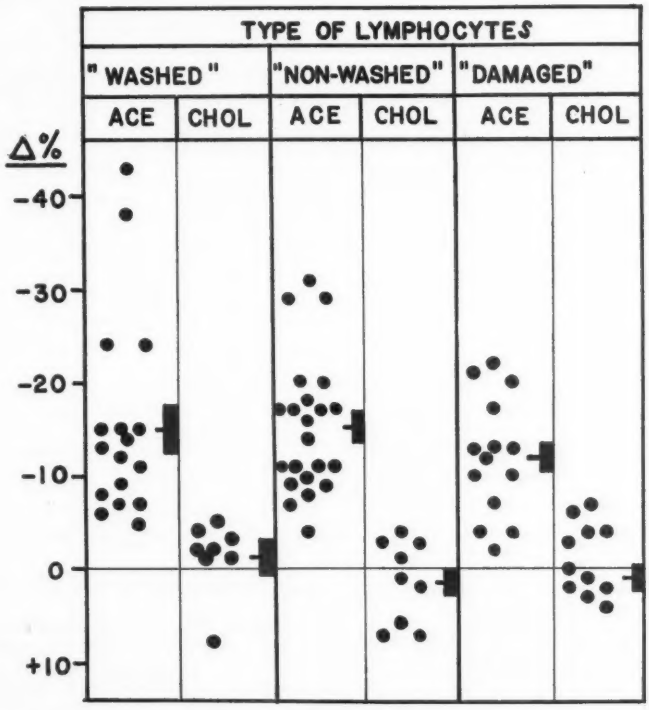

FIG. 2 illustrates the lymphocytolytic effect of 10 μg. per cc. ACE and cholesterol (CHOL) upon varying types of lymphocytes incubated for one hour at 38° C. in lymphoid homogenates. Each dot represents a single experiment; the mean of each group is shown at the line projecting from the black bar; the standard error of the mean in each group is represented by the height of the black bar.

(10 μg. per cc.) was used as a control. It will be seen that in the presence of lymphoid homogenates, ACE significantly accelerates the breakdown of all types of lymphocyte suspensions.

To determine whether the Δ% values obtained with ACE are influenced by factors such as the initial cell concentration, the lymphocyte concentration of the non-steroid control after 1 hour of

incubation and the degree of spontaneous breakdown, the individual ACE values were plotted against each of these variables and these relationships are illustrated in Figure 3. It will first be seen by the use of the $\Delta\%$ index that the lymphocytolytic activity of ACE is not significantly influenced by variations in the rate of spontaneous lymphocyte breakdown. There may be a tendency for $\Delta\%$ values produced by ACE to decline as the number of lymphocytes in the system increases. This is most evident when $\Delta\%$ ACE is plotted against log cell concentration of the control after 1 hour of incubation, and is less marked when plotted against the log of initial concentration.

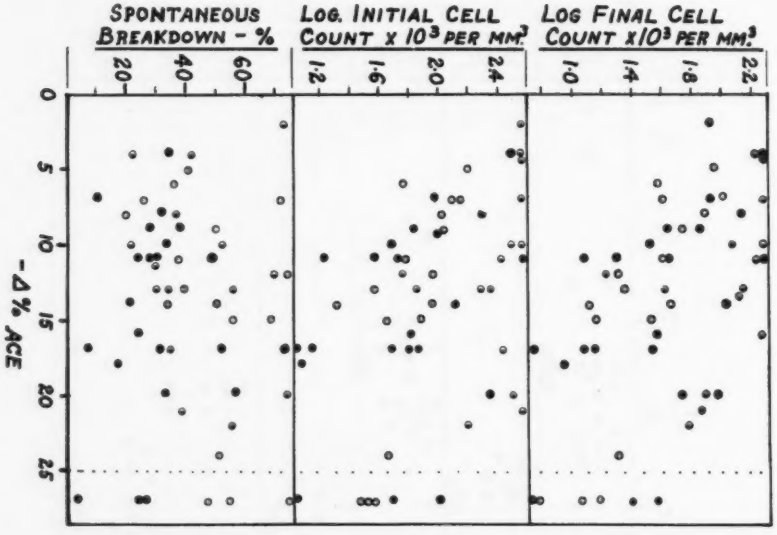

FIG. 3 illustrates the influence of spontaneous breakdown, the initial lymphocyte concentration, and the lymphocyte concentration in the control after one hour incubation (referred to as final cell concentration) upon $\Delta\%$ values obtained with ACE. Each circle represents an individual experiment wherein lymphocytes were incubated with lymphoid homogenate and ACE (10 μg. per cc.) for one hour at 38° C. The open circles (o) refer to the experiments wherein "washed" lymphocytes were employed; the filled-in circles (●) refer to "non-washed" cells, while the half-filled circles (◑) refer to the experiments using "damaged" lymphocytes. All $\Delta\%$ values greater than -25 are plotted at the right of the dotted line at an arbitrary $\Delta\%$ value of -28.

Comparison of ACE lymphocytolytic activity in various media: In the next experiments, the effect of ACE upon cells incubated in media not containing lymphoid tissue constituents was tested. In seven experiments "washed" lymphocytes from a single suspension were added to serum, and to homogenates of brain, muscle and lymphoid tissue. (In each case the homogenate represented an equivalent amount of tissue from the same rabbit.) Lymphocytolysis in the

presence and absence of ACE (10 μg. per cc.) in the various media was determined simultaneously. These results are shown in Table 2. In other studies, where ACE was tested upon lymphocytes incubated in whole blood, serum or buffered saline (pH 7.0), most of the experiments were not run concurrently with lymphoid homogenates. Thus, the composite results of these experiments are presented in Table 3.

The results shown in Tables 2 and 3 demonstrate the ACE, which is an effective lymphocytolytic agent in the presence of lymphoid homogenates, is without significant effect upon lymphocytes incubated in muscle homogenate, blood, serum or buffered saline. With

TABLE 2. COMPARATIVE LYTIC ACTIVITY OF ACE UPON "WASHED" LYMPHOCYTES INCUBATED IN SERUM AND TISSUE HOMOGENATES FOR ONE HOUR

Experiment Number	Initial Count $\times 10^3$	Serum		Lymphoid homogenate		Brain homogenate		Muscle homogenate	
		S.B.*	Δ% ACE	S.B.	Δ% ACE	S.B.	Δ% ACE	S.B.	Δ% ACE
	cells per mm.^{3}	*per cent*		*per cent*		*per cent*		*per cent*	
1	30	0	−15	82	−38	0	−18	20	− 6
2	25	34	+10	53	−43	32	−16	24	− 7
3	130.5	27	+ 7	71	− 7	36	−11	49	+14
4	108	16	−13	50	− 9	26	−21	19	− 4
5	150	39	+21	41	− 5	26	−11	49	+ 4
6	57.5	11	+ 6	36	− 6	22	− 7	41	+ 2
7	76	31	− 4	55	−15	36	−10	25	− 4
Mean		22.6	+ 1.7	55.5	−17.6	25.4	−13.4	32.5	− 0.1

* Spontaneous breakdown in the medium in the absence of steroids.

brain homogenates, as with lymphoid homogenates, ACE is active. It may be mentioned that Table 2 also demonstrates that ACE lymphocytolytic activity need not necessarily be associated with a high rate of spontaneous lymphocyte breakdown (as occurs with lymphoid homogenates) since the degree of spontaneous lymphocyte breakdown in homogenates of brain is less than that which occurs in muscle homogenates.

Nature of the Factor in Lymphoid Tissues

The differences in the lymphocytolytic activity of ACE observed with salt solutions, serum, whole blood and tissue homogenates seem best explained on the basis that homogenates of lymphoid organs and brain contain a factor which potentiates the lytic activity of ACE upon lymphocytes. Since lymphoid tissue is presumed to be the predominant site of lymphocyte breakdown *in situ*, it was of interest to obtain information concerning the nature of the factor present in lymphoid homogenates.

Thermolability of the Factor. The effect of heat treatment upon the ability of lymphoid homogenates to potentiate ACE lymphocyto-

TABLE 3. LYTIC ACTIVITY OF ACE UPON "WASHED" LYMPHOCYTES INCUBATED IN SERUM, BLOOD AND SALINE SOLUTIONS AS COMPARED TO LYMPHOID HOMOGENATES‡

Incubation media	Steroid†	Number of experiments	Mean Δ%
Lymphoid Homogenate	ACE	17	−15.0±2.7*
	CHOL.	10	− 0.3±1.4
Serum	ACE	14	− 2.0±2.6
	CHOL.	8	− 0.1±1.4
Buffered Saline	ACE	9	+ 0.1±1.7
	CHOL.	10	− 0.9±1.6
Blood	ACE	7	− 2.3±2.0
	CHOL.	6	− 1.0±1.8

* Standard error of the mean.
† Steroid concentration, 10 μg. per cc.
‡ Incubated for one hour at 38° C.

lytic activity was studied in five experiments. In each of these experiments a single "washed" lymphocyte suspension and homogenate of lymphoid tissue was employed. One portion of the homogenate was heated for 15 minutes in a steam bath; the other portion was not heated. Equal volumes of a cell suspension were then added to both types of homogenates, and the mixtures were incubated for 1 hour at 38° C., with and without ACE (10 μg. per cc.). The results of these experiments are illustrated in Table 4. It will be seen that heat treatment of the lymphoid homogenate removes its ability to potentiate the lymphocytolytic activity of ACE. The spontaneous breakdown of washed lymphocytes in lymphoid homogenate is, however, not affected by heat treatment of lymphoid tissue factors.

Influence of Incubation Temperature: To determine the influence

TABLE 4. THE EFFECT OF HEAT-TREATED HOMOGENATES AND LOWERED INCUBATION TEMPERATURE UPON THE ACE LYMPHOCYTOLYTIC REACTION

Additions to cell suspension	Incubation temperature	Number of experiments	Δ% ACE	S.B.*	
				Mean	Range
				per cent	*per cent*
ACE† +normal homogenate	38°	5	−10.6±1.07	59	(35–81)
ACE +boiled homogenate‡	38°	5	+ 2.0±2.35	58	(30–75)
ACE +normal homogenate	38°	5	−11.0±1.38	40	(25–65)
ACE +normal homogenate	5°	5	− 0.8±1.82	18	(6–25)

* S.B. Spontaneous breakdown.
† ACE, 10 μg. per cc.
‡ Homogenate placed in steam bath for 15 minutes, cooled and then rehomogenized.

of the incubation temperature upon the reaction, 10 μg. per cc. ACE plus lymphoid homogenate was incubated with "non-washed" lymphocytes at both 38° and 5° C. for one hour. The results of five experiments, where in each case the same cells and homogenate were employed, are also illustrated in Table 4. It will be seen that the lymphocytolytic activity of ACE is significantly reduced when the reaction proceeds at 5° as compared to the usual incubation temperature of 38° C. It will also be noted that the spontaneous breakdown of lymphocytes is inhibited by the lower temperature.

Fractionation of Homogenates by Centrifugation: In the next experiments, lymphoid homogenates were fractionated by centrifugation and the activity of each fraction compared to that of the whole homogenate.

Upon centrifugation of lymphoid homogenates at approximately 2,000 RPM for 30 minutes, the homogenate yields three separate phases. The *bottom* of the centrifuge tube contains the insoluble particles; in the *middle*, there is a pink-colored cloudy, soluble fraction; and at the *top* there is a small white, fatty layer. The activity of these fractions relative to the whole homogenate was studied in 5 experiments. In each experiment "washed" lymphocytes from a single suspension were used; the lymphoid homogenate was prepared by homogenizing the tissue with 0.5 per cent sodium chloride containing M/30 phosphate buffer (pH 7.0). One portion of homogenate was set aside while the other was centrifuged. The bulk of the "top" fraction was removed with a spatula and resuspended in a volume of saline-phosphate equal to the original volume of the homogenate portion subjected to centrifugation. It was not possible to remove the "top" fraction quantitatively and thus the "middle" fraction was contaminated to some degree. The "middle" fraction was then removed with a pipette; and the "bottom" fraction was washed with saline, resuspended in saline-phosphate, and brought to its original volume. In addition to ACE, the effect of cholesterol (10 μg. per cc.) was simultaneously studied.

The results of these fractionation studies are shown in Table 5 and indicate that the activity present in lymphoid homogenate tends to be concentrated mainly in the "top" fraction, although there is some activity in the "middle" fraction while the "bottom" fractions are inactive. In three experiments where the "top" fraction of lymphoid homogenates was heated for 15 minutes at 100° and then incubated with "washed" lymphocytes in the presence of ACE, lymphocytolytic activity was absent.

In summary the findings presented in this section suggest that there is present in lymphoid homogenates a heat-labile factor which significantly increases the lymphocytolytic activity of ACE. This factor is ineffective at low temperatures and appears to be associated with the "light" lipoid particles in homogenates of lymphoid tissue.

TABLE 5. VARIOUS FRACTIONS OF LYMPHOID HOMOGENATE UPON THE ACE LYMPHOCYTOLITIC REACTION

Experiment†	Initial count $\times 10^3$	Whole homogenate			Top fraction			Middle fraction			Bottom fraction		
		S.B.*	Δ% ACE	Δ% CHOL.	Δ%	Δ% ACE	Δ% CHOL.	Δ%	Δ% ACE	Δ% CHOL.	S.B.	Δ% ACE	Δ% CHOL.
	cells per mm.^{3}	*per cent*			*per cent*			*per cent*			*per cent*		
1	30.2	70	−18	−5	65	−17	+2	70	−15	−10	81	−17	− 3
2	28.5	46	−27	−4	33	−11	+5	51	−11	− 3	52	− 7	− 5
3	43.0	48	−13	−2	43	−14	+2	46	− 8	− 1	75	+ 9	+ 2
4	92.5	78	−12	+8	55	−13	−4	76	− 9	+ 1	73	−13	−14
5	99.5	55	−14	−3	46	−17	−5	61	+ 9	− 8	46	+ 7	− 3
Mean		59	−16.8	−1.2	48	−14.5	0	61	− 6.8	− 4.2	65	− 4.2	− 4.6

† "Washed" lymphocytes used.
* Refers to the percentage lymphocyte breakdown in the absence of steroids, upon incubation for 1 hour at 38° C.

Effect of Crystalline Steroids

In considering the specificity of the ACE lymphocytolytic reaction, our attention was directed primarily to three questions. (a) Is the ACE effect described non-specific in the sense that any hormonal steroid would reproduce the effect of ACE, *i.e.*, activation by lymphoid homogenate? (b) Would other steroids possess lymphocytolytic activity in the absence of the lymphoid tissue factor? (c) Can the lymphocytolytic activity of ACE be accounted for on the basis of a single crystalline corticosteroid known to be present in the whole extract, for example, Compound E of Kendell?

TABLE 6. THE EFFECT OF VARIOUS STEROIDS UPON LYMPHOCYTES INCUBATED IN SERUM OR LYMPHOID HOMOGENATES FOR ONE HOUR AT 38° C.

Steroids*	"Non-washed" lymphocytes				"Damaged" lymphocytes	
	Lymphoid homogenate		Serum		Lymphoid homogenate	
	Number	Mean Δ%	Number	Mean Δ%	Number	Mean Δ%
ACE	22	*−15.8*†±1.6‡	13	− 5.3±1.9	13	*−12.0*±1.6
α-Estradiol	10	*−12.6* ±1.8	15	*−11.5*±1.7	—	
17-hydroxyprogesterone	6	*− 8.3* ±2.0	9	*− 7.1*±2.0	—	
Ethyl testosterone	6	− 2.2 ±2.3	12	*−12.4*±2.8	—	
Desoxycorticosterone acetate	10	− 2.1 ±2.3	10	− 4.2±3.8	6	− 0.8±2.5
Desoxycorticosterone	4	+ 1.7 ±0.8	—		6	− 2.7±2.4
Corticosterone	10	− 1.3 ±2.9	12	− 5.0±2.6	6	+ 4.3±3.1
Compound A	11	− 1.1 ±3.4	14	− 5.5±3.4	6	− 0.7±2.1
Compound E	4	− 0.3 ±2.1	—		6	+ 1.8±2.6
Androsterone	6	+ 1.8 ±3.6	6	− 0.2±1.7	—	
Cholesterol	8	+ 1.5 ±1.5	12	− 1.2±1.4	11	+ 1.1±1.1

* Steroid concentration 10 μg. per cc.
† All values which are italicized are statistically significant ($p < 0.05$) as compared to the appropriate cholesterol control group.
‡ Standard error of the mean.

To obtain preliminary information on these points a number of crystalline steroids, both corticoid and non-corticoid were tested at a single dosage level of 10 μg. per cc. upon lymphocytes incubated for one hour at 38° C. Table 6 presents the collected data of experiments where various steroids were tested upon "non-washed" and damaged lymphocytes incubated in either serum or lymphoid homogenates. The data may be summarized briefly as follows:

(1) None of the steroids tested exhibit the characteristic response induced by ACE; i.e. inactivity in serum, activation by lymphoid homogenate.

(2) Of the crystalline cortical hormones tested, some of which are known to be present in ACE, none possess significant lymphocytolytic activity at a dosage level of 10 μg. per cc. in the presence of either lymphoid homogenate or serum.

(3) Of the four non-cortical hormonal steroids tested, only androsterone is without any significant lymphocytolytic activity. Estradiol is equally effective as a lymphocytolytic agent upon cells incubated either in homogenates or serum. 17-hydroxy progesterone, although somewhat less active, behaves similarly. Ethyl testosterone is significantly effective against lymphocytes incubated in serum, but in-

effective in the presence of homogenates, a situation which is the reverse of that found with ACE.

To rule out the possibility that the results with estradiol upon "non-washed" cells incubated in serum are due to the possible presence of the lymphoid tissue factor present as a contaminant in "non-washed" cell suspensions, a second series of eight experiments with estradiol using "washed" lymphocytes was performed. The results obtained were essentially similar to those using "non-washed" cells the mean $\Delta\%$ of estradiol being -12.9 ± 1.8 upon "washed" cells.

DISCUSSION

In accord with the working hypothesis upon which this study was initiated, it has been found that ACE is an effective lymphocytolytic agent in the presence of homogenates of lymphoid tissue, but that ACE is ineffective in serum, blood or buffered saline solutions. Preliminary studies suggest that the lymphoid tissue co-factor is enzymatic, since heat treatment of homogenates or decreasing the reaction temperature abolishes the ability of lymphoid homogenates to potentiate ACE lymphocytolytic activity. Fractionation studies of lymphoid homogenates by centrifugation indicate that the ACE co-factor is associated with the "light lipoid" fraction of homogenates; however, the question of whether this association is real or merely an artifact remains to be determined.

The lymphocytolytic activity obtained with ACE in the presence of lymphoid homogenates, while small, is highly significant statistically. Moreover, the lymphocytolytic activity of ACE was consistently observed in experiments using three different types of lymphocyte suspensions. Considering the results with the "washed" and "non-washed" lymphocytes, ACE produced an average $\Delta\%$ value of -15, which means that 15 cells per 100 cells of the control were broken down by ACE during one hour of incubation. This value is equivalent to increasing the percentage lymphocyte breakdown per hour from an average value of 38 per cent in the absence of ACE, to 48 per cent in the presence of ACE. While these figures of ACE action appear small, a $\Delta\%$ value of -15 in a system containing 4.0 cc. total volume, where the control contains 100,000 cells per mm^3, represents the breakdown of 6×10^7 lymphocytes by 40 μg. of ACE in one hour. When it is considered that the limiting factor in the reaction described may be the concentration of lymphoid co-factor and that lymphocytolysis was measured at a single arbitrary interval (which may not give maximal results) it is apparent that in future experiments greater *in vitro* effects of ACE may well be achieved.

Contrary to expectations, it was observed that homogenates of brain likewise potentiate ACE lymphocytolytic activity. It thus seems clear that the ACE co-factor hypothesized is not present exclusively in lymphoid tissue. The distribution of similar factors in other tissues awaits further experimentation. It should be empha-

sized, however, that the ACE accessory factor need not be distributed generally throughout the body tissues, since homogenates of muscle are completely inactive in potentiating ACE. The presence of an accessory factor for ACE lymphocytolytic activity *in vitro* in lymphoid tissue, a major site of lymphocyte breakdown *in situ*, would appear to have clear physiological significance for an understanding of ACE action upon lymphocytes. On the other hand, the presence of an ACE co-factor in brain is, as yet, completely inexplicable. The nature of the co-factor in brain, its relationship to the lymphoid tissue factor and its physiologic importance remains to be determined.

At the present time, nothing is known of the manner of action of the co-factors described. It would appear that they could act either by increasing the sensitivity of lymphocytes to ACE or contrariwise, by transforming ACE from an inactive to a potent lymphocytolytic agent. The available data does not permit differentiation between these basically dissimilar mechanisms. However, the finding that some steroids, such as estradiol, exhibit significant lymphocytolytic activity in the absence of lymphoid homogenates, may perhaps be taken as suggestive evidence favoring the view that the co-factors (enzymes?) transform one or more of the steroids in ACE to another compound which is the active agent. The idea that lymphoid homogenates act by transforming steroids rather than by altering the sensitivity of lymphocytes could likewise account for the finding that ethyl-testosterone, active against lymphocytes in serum, is inactive as a lymphocytolytic agent in the presence of lymphoid homogenates.

When steroids such as ACE or estradiol accelerate the rate of breakdown of lymphocytes incubated in lymphoid homogenate, they are producing this result upon cells whose cytoplasmic membrane is no longer functional, as evidenced by the fact that such cells are freely permeable to eosin. It thus seems unlikely that lymphocytolytic steroids act by altering the cytoplasmic membrane. The lymphocyte is largely nuclear, and lymphocytolysis as measured by our method in reality measures dissolution of nuclei, composed largely of desoxyribonucleoprotein complex (Mirsky and Pollister, 1946). It thus seems legitimate to consider the possibility that steroid hormone action upon lymphocytes involves an effect upon desoxyribonucleoproteins possibly via desoxyribonuclease or through other means.

In considering the steroid specificity aspects of the ACE lymphocytolytic reaction, it is apparent that our preliminary studies of crystalline steroids are incomplete since only a single dosage level was employed under arbitararily selected conditions which had proved satisfactory for the demonstration of ACE activity. If other dosage levels had been tested in a manner designed to test activity in a kinetic fashion, it is possible that the results might be qualitatively different from those reported here. It is therefore apparent that it is not profitable to discuss these incomplete *in vitro* findings in relation to the known *in vivo* actions of the "carbohydrate-active" cortico-

steroids upon lymphoid tissue. Nevertheless, certain aspects of the steroid specificity studies are worthy of mention. Of the ten crystalline steroids tested not a single compound reproduced the results obtained with ACE (i.e. *inactivity* in serum, *activity* in the presence of lymphoid homogenate). To this extent, the results obtained demonstrate a degree of specificity for the *in vitro* lymphocytolytic reaction of ACE described. It is also of some interest that estradiol, ethyl testosterone and 17-hydroxyprogesterone all exhibit significant lymphocytolytic activity in the absence of the lymphoid cofactor, suggesting that these steroids act directly upon lymphocytes. While these non-corticoids can be differentiated from ACE in that they are not activated by lymphoid homogenates (ethyl testosterone is presumably inactivated by a factor present in lymphoid homogenates) it seems striking that these steroids should act upon lymphocytes *in vitro*, while corticosteroids like corticosterone, Compound A and Compound E are inactive. Is it possible that the *in vivo* specificity of hormone action upon lymphoid tissue is determined not so much by the specific configuration of the steroid molecule, but by factors (e.g. capillary and cell permeability) which determine the entry of steroids into the target organ in question?

The negative findings with crystalline corticosteroids tested indicate that the action of the ACE preparation tested cannot be explained on the basis of a single corticosteroid of the types employed. This suggests that ACE may contain a highly potent, as yet unidentified substance which is quantitatively dissimilar from the types of corticosteroid tested, or it may be that the lymphocytolytic activity of ACE is the result of synergistic action of two or more of the crystalline hormones tested?

It should not be inferred from these studies that lymphocytolytic activity is the only, or indeed the most important, action of adrenocortical steroids upon lymphocytes. The suggestion of Robertson (1948) that ACH controls the maturation and mitotic rate of young lymphocytes, the histological observation that ACH action *in vivo* may produce cytoplasmic dissolution of lymphocytes leaving the nucleus intact (White and Dougherty, 1946); and the findings of Schrek (personal communication) that ACE (in the absence of added lymphoid homogenate) increases lymphocyte permeability to eosin, without promoting cell lysis, may be taken to indicate that ACH affects not one but several factors involved in maintenance of the structural integrity of the lymphocyte.

One aspect of our results, although not directly related to steroid action upon lymphocytes, merits special attention. It should be noted that in the absence of added steroids, lymphoid homogenates exhibit marked lymphocytoxic activity as evidenced by alteration of permeability, shrinkage and accelerated dissolution of lymphocytes. The toxic activity responsible for these effects appears to be a separate factor from that which potentiates ACE lymphocytolytic action.

Preliminary studies indicate it to be thermostable and absent in muscle and brain homogenates. The finding of a highly active lymphocytotoxic factor in lymphoid tissue appears to warrant further study.

SUMMARY

Under conditions where spontaneous lymphocytolysis is proceeding, it has been observed that ACE (10 μg. per cc.) in the presence of lymphoid tissue homogenates significantly increases the rate of lymphocyte breakdown. ACE is likewise active with brain homogenates but inactive in the presence of homologous serum, or defibrinated blood, buffered salt solutions, and muscle homogenates. The factor present in lymphoid tissue homogenates may be an enzyme and appears to be associated with the light-lipoid particles of the homogenate. The lymphocytolytic reaction obtained with ACE seems to be specific in the sense that none of the ten crystalline steroids tested duplicate the results obtained with ACE. Estradiol, 17-hydroxy progesterone and ethyl testosterone all exhibit lymphocytolytic activity upon cells incubated in serum. None of these steroids, however, exhibit an increased lymphocytolytic activity with lymphoid homogenates and one of these steroids (ethyl testosterone) is inactive under these conditions. In the dosages tested, 11-dehydrocorticosterone. 17-hydroxy 11-dehydro corticosterone, corticosterone, 11-desoxycorticosterone (or its acetate) and androsterone do not exhibit lymphocytolytic activity either in the absence or presence of lymphoid homogenates. The limitations and possible significance of these *in vitro* findings are briefly discussed.

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THE EFFECT OF PREGNENOLONE ON REINITIATION AND MAINTENANCE OF SPERMATOGENESIS IN HYPOPHYSECTOMIZED RATS[1]

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Δ^5-PREGNENOLONE has recently been isolated from hog testicular extract (Haines, Johnson, Goodwin and Kuizenga, 1948). This steroid can maintain spermatogenesis in hypophysectomized rats (Leathem and Brent, 1943) and in estrogen treated rats (Albert and Selye, 1942). However, it is unable to reinitiate spermatogenesis in the atrophic testis following hypophysectomy or estrogen therapy (Masson, 1946). It seemed of interest to determine whether larger dosages or longer treatment periods would reinitiate spermatogenesis in hypophysectomized rats. The effect of pregnenolone on the maintenance of spermatogenesis also was restudied, using small dosages of hormone. Subcutaneous versus oral routes of administration where compared. The effect of small dosages of pregnenolone on testis and accessory sex glands of normal rats was also studied.

METHODS

Adult male rats of the Long-Evans strain were hypophysectomized and 20 days later received daily subcutaneous injections for 20 days of either 3 mg. or 20 mg. of pregnenolone or 4 mg. of pregnenolone acetate or 3 mg. of testosterone propionate.[3] The left testis and epididymis were removed at the beginning of treatment and served as controls. In the maintenance experiments treatment with daily subcutaneous injections of 1 mg. of pregnenolone or 2 mg. of pregnenolone acetate or daily force feedings with 10 mg. of pregnenolone in aqueous suspension was started at the time of operation and continued for 20 days. Normal adult male rats were injected subcutaneously each day for 20 days with 0.1 mg. or 0.5 mg. or 2.5 mg. of pregnenolone.

At autopsy, the testes and sex accessory glands were weighed to the nearest milligram and fixed in Bouin's fluid. Sections were made at 8 μ and stained with hematoxylin and eosin. The pituitary capsules were serially sectioned, and only completely hypophysectomized animals are reported.

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[3] Pregnenolone and Pregnenolone Acetate and Testosterone Propionate were generously supplied by the Schering Corporation.

RESULTS

Neither pregnenolone, in dosage up to 20 mg. per day, nor pregnenolone acetate were able to reinitiate spermatogenesis nor prevent the progressive decrease in testis or sex accessory gland weight during the period of 20 to 40 days following hypophysectomy (table 1). Testosterone propionate, under similar conditions, increased testis and sex accessory gland weight (table 1), and served to increase the number of cell layers and the diameter of the tubules of the testis. Spermatozoa did not form during the 20 day treatment period, however.

As little as 1 mg. of pregnenolone or 2 mg. of pregnenolone acetate subcutaneously per day maintained spermatogenesis for 20 days fol-

TABLE 1. WEIGHTS (MG.) OF TESTES, EPIDIDYMIDES, SEMINAL VESICLES AND VENTRAL PROSTATE OF ADULT RATS TREATED WITH THE INDICATED MATERIALS BY DAILY SUBCUTANEOUS INJECTION FOR 20 DAYS STARTING 20 DAYS AFTER HYPOPHYSECTOMY. AT THE START OF TREATMENT THE LEFT TESTIS AND EPIDIDYMIS WERE REMOVED AND SERVED AS CONTROLS

Treatment	No. of animals	Testis Wt. Avg. and range		Epididymis Wt. Avg. and range		Seminal vesicles Avg. and range	Ventral prostate Avg. and range
		Before Tr.	After Tr.	Before Tr.	After Tr.		
Sesame Oil 0.75 cc./d.	1	333	155	109	84	52	15
Pregnenolone 3 mg./d.	2	343 (301–384)	164 (142–185)	88 (74–101)	80 (75–84)	55 (45–64)	9 (6–11)
Pregnenolone 20 mg./d.	2	249 (238–260)	164 (162–185)	76 (74–77)	63 (61–64)	35 (34–36)	8 (6–10)
Pregnenolone Acetate 4 mg./d.	3	299 (277–334)	134 (125–148)	75 (75–76)	65 (62–70)	57 (40–70)	10 (8–13)
Testosterone Propionate 3 mg./d.	2	239 (190–288)	309 (247–370)	84 (68–95)	198 (195–200)	299 (290–307)	229 (205–252)

lowing hypophysectomy and partially maintained testis weight (table 2). Seminal vesicle, prostate and adrenal weights were not maintained and resembled those of hypophysectomized controls. Epididymis weight was partially maintained (table 2). Oral administration of 10 mg. of pregnenolone per day was completely ineffective in maintaining spermatogenesis, and testis weight was similar to that of controls (table 2).

The administration of 0.1 mg. or 0.5 mg. or 2.5 mg. of pregnenolone per day for 20 days to normal rats failed to significantly alter testis, sex accessory gland or adrenal or pituitary gland weight. Testis structure remained completely normal.

DISCUSSION

The presence of a steroid in testis tissue which is effective in maintaining spermatogenesis and which is completely devoid of androgenic ability is of considerable theoretical importance. It raises

TABLE 2. WEIGHTS (MG.) OF TESTES, SEX ACCESSORY GLANDS AND ADRENALS OF ADULT HYPOPHYSECTOMIZED RATS TREATED DAILY FOR 20 DAYS FOLLOWING OPERATION WITH SUBCUTANEOUS INJECTIONS OF 1 MG. OF PREGNENOLONE IN 0.25 CC. SESAME OIL OR 2 MG. OF PREGNENOLONE ACETATE IN 0.25 CC. OF SESAME OIL, OR 0.25 CC. SESAME OIL ALONE, OR FORCE FED WITH 10 MG. OF PREGNENOLONE IN 2.0 CC. OF WATER

Treatment	No. of animals	Testes		Epididymides		Seminal vesicles		Ventral prostate		Adrenals	
		Av.—Range	Contr. =1.00	Av.—Range	Contr. =1.00	Av.—Range	Contr. =1.00	Av.—Range	Contr. =1.00	Av.—Range	Contr. =1.00
Pregnenolone 1 mg./d. subcutaneously	3	1734 (1368–2383)	2.12	403 (304–550)	1.56	87 (66–98)	1.00	25 (15–32)	1.14	13 (12–14)	1.00
Pregnenolone Acetate 2 mg./d. subcutaneously	1	1240	1.52	351	1.35	80	0.92	22	1.00	11	0.85
Oil Injected Controls	2	818 (700–936)	1.00	259 (224–295)	1.00	87 (75–98)	1.00	22 (21–23)	1.00	13 (13–14)	1.00
Pregnenolone 10 mg./d. orally	4	777 (695–868)	0.95	236 (195–263)	0.91	84 (65–93)	0.97	26 (24–28)	1.09	14 (14–15)	1.08

the possibility that pituitary F.S.H. action on the tubules may be mediated by the release of such a steroid which would have no influence on the accessory sex glands.

Though most steroids which are able to maintain spermatogenesis in hypophysectomized rats are also androgens, there is no definite correlation between these actions (Nelson, 1937). Even weakly androgenic progesterone has spermatogenesis maintaining ability (Nelson, 1938; Seyle and Friedman, 1941).

The absence of any inhibitory effect of pregnenolone on pituitary gonadotrophin secretion, as judged by the lack of effect of small dosages on normal testis and accessory gland weight and structure, is also of considerable interest.

The ability of pregnenolone to maintain spermatogenesis contrasts with its inability to reinitiate spermatogenesis. This confirms Masson's (1946) findings, and is similar to the early reports with testosterone (Cutuly, McCullagh and Cutuly, 1938; Hamilton and Leonard, 1938). Later experiments, using larger dosages of testosterone for longer periods (Nelson, 1940, 1941, 1946), or small dosages administered by intratesticular pellet implant (Dvoskin, 1947), demonstrated testosterone to be effective in reinitiating spermatogenesis in hypophysectomized rats. In the present experiments, testosterone failed to reinitiate spermatogenesis in a 20 day period, though it definitely increased testis weight and caused an increase in the size and number of cells layers in the tubules. This did not occur in the pregnenolone treated rats, and may remotely be due to the short (20 day) treatment period, though some effect should be expected in this time.

Pregnenolone has also recently been found to be without effect on sperm count in human infertility (Tyler, Payne and Kirsch, 1948).

CONCLUSIONS

Neither pregnenolone in dosages of 3 mg. or 20 mg. per day or pregnenolone acetate in dosages of 4 mg. per day were able to reinitiate spermatogensesis or prevent progressive testis weight loss during a 20 to 40 day period after hypophysectomy.

Small dosages of pregnenolone (1 mg. per day) or pregnenolone acetate (2 mg. per day) subcutaneously administered were able to maintain spermatogenesis and partially maintain testis weight for 20 days after hypophysectomy. Sex accessory gland weight, except for epididymis, were similar to untreated hypophysectomized controls.

Orally administered pregnenolone in dosage of 10 mg. per day was completely ineffective in maintaining spermatogenesis in hypophysectomized rats.

Pregnenolone in dosage of 0.1 mg. or 0.5 mg. or 2.5 mg. per day had no effect on testis or sex accessory gland weight in normal rats.

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THE ANTIGOITROGENIC ACTION OF ELEMENTAL IODINE

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DVOSKIN (1947 a and b) has presented evidence that elemental iodine injected into rats can be converted into thyroxin or thyroxin-like material in tissues other than the thyroid gland. The observations presented here give some support to this hypothesis, but our experimental results differ so greatly from those of Dvoskin that they seem worth reporting.

In Dvoskin's experiments, the injection of elemental iodine prevented entirely the goitrogenic action of thiouracil given orally or by implantation. In our experiments elemental iodine given by injection had only a limited antigoitrogenic action which was no greater than that of iodides given orally. Evidence of extra-thyroidal formation of thyroxin-like material from elemental iodine was apparent only in experiments on metabolic rate.

METHODS

White male rats of a Wistar strain, weighing between 135 and 175 gm. were given thiouracil 0.1% in drinking water. Eight rats were used in each group (Table 1). One control group received thiouracil only. Another control group received (in addition to thiouracil) daily subcutaneous injections of mineral oil. Five other groups received daily subcutaneous injections of various amounts of elemental iodine in mineral oil. The animals were killed after 14 days and the thyroid glands were removed and weighed. In some experiments propylene-glycol was used as the solvent for iodine and one experiment was performed with iodine trichloride.

The experiments on oxygen consumption were done by a closed circuit technique, using a battery of eight chambers in a water-bath at 30°C., with one rat in each chamber. All the animals received thiouracil (0.1% in drinking water). Four of each group of eight were injected daily with iodine in mineral oil (1 mg. in 0.2 cc.). Measurements were made at the same time each morning, six days a week, on all eight rats simultaneously, without preliminary fasting. The figure used for the initial metabolic rate was the average of five measurements made before treatment was started. The results were calculated as cc. of oxygen per sq. meter of body surface per minute. The final figure was the average of the last five days of fifteen days of treatment. During the last five days of this period the rate of change of metabolic rate

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was perceptible on inspecting the figures, but small enough to justify averaging them. The results are presented in Table 2 as per cent decrease in oxygen consumption. The per cent difference for each animal was treated as a single figure and the S.E. shown in Table 2 indicates the variability among the four animals in each group. The rats used weighed between 125 and 180 gm.

OBSERVATIONS

It is apparent that in our experiments elemental iodine by injection did not prevent the goitrogenic action of thiouracil. The diminution in enlargement which occurred was only of the order of magnitude

TABLE 1. ANTIGOITROGENIC ACTION OF ELEMENTAL IODINE BY INJECTION. DURATION TREATMENT 2 WEEKS

No.	Experimental groups	Thyroid wt. mg./100 gm. ±S.E
1	Controls (Thiouracil only, no injections)	26.5±1.5
2	Controls (Thiouracil+0.2 cc. oil injected)	27.8±1.6
3	I_2 5 mg./d. in 0.2 cc. of oil	22.7±1.6
4	I_2 mg./d. in 0.2 cc. of oil	24.5±0.9
5	I_2 1 mg./d. in 0.2 cc. of oil	25.3±1.6
6	I_2 0.5 mg./d. in 0.2 cc. of oil	24.2±1.5
7	I_2 0.1 mg./d. in 0.2 cc. of oil	22.7±1.4
8	I_2 5 mg./d. in 0.2 cc. of propylene glycol	23.4±1.4
9	I_2 1 mg./d. in 0.2 cc. of propylene glycol	24.2±1.6
10	ICl 1 mg./d. in 0.2 cc. of propylene glycol	20.9±0.7
11	Untreated normals	10.2±0.4

which we have reported elsewhere as resulting from iodides given orally (Ferguson and Sellers 1948) and which has been often observed by other investigators.

The thyroid glands from the animals used in the experiments on oxygen consumption were also excised and weighed, and showed practically the same degree of partial antigoitrogenic action by injected iodine as shown in Table 1.

TABLE 2. EFFECT OF ELEMENTAL IODINE AND THIOURACIL ON OXYGEN CONSUMPTION. FOUR RATS PER GROUP; 1.0 MG. OF IODINE DAILY FOR 15 DAYS

Expt. No.	Average per cent decrease in O_2 consumption±S.E.	
	Thiouracil only	Thiouracil plus iodine
1	15.0±1.8	−0.7±1.7
2	30.0±1.7	18.5±3.1
3	15.6±3.5	5.4±2.9

From Table 2, however, it appears that elemental iodine does materially alter the metabolic response to thiouracil, as though the injected iodine had been partly converted to thyroxine. The injected iodine in one case maintained, and in the other cases partially sustained the rate of oxygen consumption. This result differs from the effect of iodides given orally which did not affect the decline of oxygen

consumption resulting from the administration of thiouracil (Ferguson and Sellers 1948). Although none of our animals showed ulceration or excessive induration at the sites of injection, the possibility that local irritation might increase the metabolic rate and thus explain our results could not be disregarded. However, when four rats which had been receiving propylthiouracil for several months were injected subcutaneously with 0.2 cc. of 5% aqueous formalin, producing a comparable indurated lesion, the metabolic rates actually decreased slightly.

DISCUSSION

Although our observations are consistent with Dvoskin's hypothesis, the thyroxin-like action of injected iodine was much weaker than in Dvoskin's experiments, and evident only in experiments on metabolic rate. Metabolic experiments were not reported by Dvoskin. Two possible explanations suggest themselves. (1) Our rats were all older and larger than those used by Dvoskin. It is possible that the capacity for producing thyroxin extra-thyroidally decreasses with age. (2) It is possible that genetic differences in our animals may be responsible for our differing results.

Since these experiments were done, Barker and Lipner (1948) have reported that the protein bound iodine and the metabolic rate of thyroidectomized rats and rats treated with thiouracil increase when elemental iodine is injected. There is thus considerable evidence that thyroxin-like material may be formed outside the thyroid gland.

SUMMARY

Elemental iodine by injection did not prevent the enlargement of the thyroid gland produced in white rats by thiouracil given orally.

A limited antigoitrogenic action resulted from elemental iodine by injection but the magnitude was no greater than that often observed with iodides given orally.

Elemental iodine by injection lessened the rate of decrease of metabolic rate under treatment with thiouracil, while iodides had no such action. This observation is consistent with the hypothesis that some thyroxin-like material can be produced from elemental iodine in extra-thyroid tissues.

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INACTIVATION OF POSTERIOR PITUITARY ANTIDIURETIC HORMONE BY THE LIVER[1]

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IT HAS long been recognized that the liver bears some important relation to water metabolism (reviewed by Pick, 1929 and 1946; Ratnoff and Patek, 1942). In experimental liver damage (Adlersberg, 1934; Adlersberg and Fox, 1943) and in liver disease of man (Labby and Hoagland, 1947; Ralli *et al.*, 1945; Leslie *et al.*, 1948) water retention has been reported. The presence of antidiuretic substances in urine has been associated with liver dysfunction (Ralli *et al.*, 1945; Leslie and Ralli, 1947; Hall, Frame and Drill, 1949). The suggestion has been made that this may be the well known neurohypophyseal antidiuretic hormone (ADH) appearing in the urine because it is not effectively inactivated by the damaged liver (Ralli *et al.* 1945).

ADH is inactivated at some body site as judged by the small amounts excreted after injection (Heller and Urban, 1935; Ingram, Ladd and Benlbow, 1939) and the *in vitro* studies of Heller and Urban (1935) indicate that while kidney and blood inactivate ADH, liver tissue is more effective.

To test the role of the liver in the inactivation of posterior pituitary antidiuretic hormone, we have studied the response of animals to Pitressin administered through an hepatic portal drainage as compared with the response of those which received the hormone by other routes. This technique has been effectively employed in studying the role played by the hepatic tissue in the inactivation of other hormones. In addition, the antidiuretic activity of Pitressin was tested after incubation with extracts of several different tissues.

Some of these observations were recorded in an earlier abstract (Eversole, Birnie and Gaunt, 1948).

MATERIAL AND METHODS

Mature male rats weighing approximately 200 grams were used in the water excertion tests. They were fasted but allowed water for 18 hours prior to testing. At the end of the fast, one dose of distilled water amounting to 4 cc. per 100 sq. cm. of body surface was warmed to 100°F and administered

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by stomach tube. The dose for a 200 gram rat was 12.5 cc. with body surface calculated according to the formula of Benedict (1938). The animals were placed in individual metabolism cages and the urine output measured at 30 minute intervals for a period of 3 hours. Since there is a day to day variation in the diuretic response to water, equal numbers of controls were run simultaneously with experimental animals.

All animals were anesthetized with ether and in those receiving intrasplenic injections, the spleen was withdrawn from the body cavity through a subcostal incision. In the experimental rats, injections of 0.1 cc. of freshly-diluted Pitressin solution was made directly into the exposed spleen with a 27 gauge hypodermic needle three-quarters of an inch long. In addition, 0.1 cc. of water was given subcutaneously because control animals received Pitressin under the skin. Immediately after injection, the spleen was returned to the body cavity and the incision sutured. Control animals were given Pitressin subcutaneously or intramuscularly in amounts equal to that given the experimental cases. In addition, the spleen of controls was exposed and manipulated as above and injected with 0.1 cc. of water.

For intrahepatic injections the animals were anesthetized with ether, and the left lateral lobe of the liver forced gently through a midventral incision. Pitressin injections were made into the exposed liver lobe, the incision sutured and 0.1 cc. of water was given subcutaneously. Control animals were injected subcutaneously with Pitressin and with water intrahepatically.

Pitressin was given 30 minutes before or 30 minutes after the oral administration of the test dose of water. Hormone and water could not be given satisfactorily at the same time because the animals had to be anesthetized for the hepatic and splenic injections and water administered to etherized rats backs up from the stomach, preventing control of dosage.

For the *in vitro* studies, extracts were prepared from tissues immediately after their removal from rats killed by a blow on the head. The extracts were prepared by grinding 1 gram of tissue in a mortar with sand and extracting with 10 cc. of 1 per cent saline for 10 minutes. The homogenates were centrifuged at 2,600 R.P.M. for 10 minutes and the supernatant fluids tested for their ability to inactivate Pitressin. The extracts were adjusted to pH 6.8, the desired amount of Pitressin added, and incubated for 20 minutes at 37°C. Following incubation, 1 cc. of this material was injected intraperitoneally into hydrated animals and tested by the method of Birnie *et al.*, (1949) to determine the amount of antidiuretic material present.

RESULTS

The injection of 40 milliunits of Pitressin into the spleen was less effective in suppressing diuresis than equal doses administered by subcutaneous injection. The findings summarized in series 1 of Table 1 are from animals receiving Pitressin 30 minutes before the water test. The differences were apparent throughout the 3 hours of observation and were shown to be statistically significant at the 90 minute interval ($P = .02$).

In the groups shown in series 2 and 3 of Table 1, the Pitressin was injected 30 minutes after water administration. Under these circumstances the antidiuretic effects of posterior pituitary extract were

more pronounced by all routes of administration than when given before the water. In series 2, intramuscular injections were markedly more effective than those given in the spleen. In series 3 in which the hormone was injected directly into the liver, its antidiuretic effects seemed to be reduced as compared with controls but no significant difference was established.

TABLE 1. ANTIDIURETIC RESPONSE TO 40 MILLIUNITS OF PITRESSIN GIVEN BY DIFFERENT ROUTES

Series	Route of administration of pitressin	No. rats	Percentage water excreted at indicated minutes					
			30	60	90	120	150	180
	Pitressin given 30 minutes prior to water							
1	Intrasplenic	21	0.4	12.0	41.8	72.4	88.6	94.9
	Subcutaneously	18	0.0	5.6	29.1	63.7	81.6	87.4
	Pitressin given 30 minutes after water							
2	Intrasplenic	6	5.9	6.4	18.3	41.8	70.0	83.1
	Intramuscular	6	0.7	0.7	8.2	28.1	50.4	67.9
3	Intrahepatic	5	0.0	3.3	25.1	49.2	73.4	81.5
	Subcutaneously	5	0.0	2.4	15.1	41.8	65.8	79.7

Smaller doses of Pitressin (10, 5, and 2 milliunits) were also used but variability was great and significant differences between subcutaneous and intrasplenic administration were not demonstrated. A more sensitive testing procedure might well reveal true differences although it is also possible that an exhibition of the effect is dependent upon a fairly high concentration of the hormone in body fluids.

TABLE 2. DISTRIBUTION OF PITRESSIN-INACTIVATING SYSTEM IN RAT TISSUE EXTRACTS (20 MILLIUNITS OF PITRESSIN ADDED TO EACH CC. OF TISSUE EXTRACT)

Tissue	No. cases	Percentage water excreted at 60 minutes
Liver	15	58.0
Kidney	6	22.6
Whole blood	6	11.3
Skeletal muscle	6	8.4
Heated liver extract*	6	1.7

*. Heated to 80° C. for 5 minutes.

In vitro studies revealed that systems capable of inactivating Pitressin were present in cell-free extracts of rat liver, kidney and blood. The greatest activity per gram of tissue was found in liver extracts (Table 2). In this table a high percentage of water excretion is indicative of Pitressin inactivation while active Pitressin causes an inhibition of diuresis.

DISCUSSION

The experiments reported here show that when 40 milliunits of Pitressin is injected into a site with hepatic portal drainage, it is less effective than when introduced into the general circulation. Such results are most easily interpreted by the theory that the liver has an important role in the inactivation of circulating posterior pituitary antidiuretic hormone. The previous finding of Heller and Urban (1935) that homogenates of liver tissue rapidly inactivate posterior pituitary extract and the demonstration here that cell-free extracts of liver have the same action, provides further evidence for this view. This does not mean that the liver is the sole site of inactivation of the antidiuretic hormone. Our *in vitro* work and that of Heller and Urban indicate that other organs inactivate Pitressin but that they are less effective than the liver.

The action of Pitressin introduced directly into the liver differed less from controls run simultaneously than did that injected into the spleen. This is perhaps due to the fact that absorption of the material could be directly into efferent vascular channels of the liver. In that case it might be exposed less to the action of liver tissue.

We have no satisfactory explanation for the failure to demonstrate differences in effectiveness of small doses of Pitressin (10, 5 and 2 milliunits) when given by intrasplenic as compared with other injection routes. There is the possibility that the test methods used were sensitive enough to detect only those effects resulting from high initial blood levels of the material.

Our results would be consistent with the hypothesis (Labby and Hoagland, 1947; Ralli *et al.*, 1945) that the water retention seen in human liver disease is due to a decreased rate of inactivation of the antidiuretic hormone by damaged liver tissues. We could not explore this idea experimentally because of inability to produce in rats a liver damage characterized by water retention. Until such experiments can be done, no definite conclusions are warranted concerning the relation of our findings to the effects of liver disease. The diseased liver itself may be producing antidiuretic materials (Shorr and Zweifach, 1948).

SUMMARY

When 40 milliunits of Pitressin were injected into the spleen of hydrated rats the antidiuretic effect was less than when the injections were made subcutaneously or intramuscularly. Such differences were not seen at lower dose levels.

Cell-free extracts of liver tissue inactivated Pitressin more effectively than extracts of kidney or whole blood.

These results indicate that the liver is one site of inactivation of the posterior pituitary antidiuretic hormone.

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THE EFFECT OF TOTAL BODY X-RADIATION ON 17 KETOSTEROID EXCRETION IN DOGS[1]

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ATTENTION HAS been drawn to alterations in adrenal cortical function in response to x-radiation (Narat, 1922; Jenkenson and Brown, 1944; Desjardins, 1928; Weichert, 1942; Hirsch, 1922; Selye, 1946). Histological and chemical alterations in the cortex in response to X-ray have been noted (Englestad and Torgerson, 1939; Weichert, 1942; Ellinger, 1948). Metabolic studies on patients exposed to X-ray have shown electrolyte changes which may be related to altered adrenal function (Goldman, 1943; Robertson, 1943). Several investigators have noted the absence of the indirect lymphopenic response to X-radiation in adrenalectomized animals (Leblond and Segal, 1942; Halberstaedler and Ichowicz, 1947; Dougherty and White, 1946). Lead shielding of the adrenal has been reported as having a positive effect on reducing the mortality to X-ray in a small number of male rats (Craver, 1946). Patt, *et al.*, (1947) have shown changes in the adrenal cholesterol content and adrenal weight following LD_{50} and higher doses of radiation. More recently they have shown this response can be prevented by hypophysectomy (Patt, 1948). Cortical replacement therapy of radiation sickness has been reported (Ellinger, 1947; Ellinger, 1948; Ellinger, *et al.*, 1949; Weichert 1942).

As the foregoing studies were designed to be carried out on patients with disease, adrenalectomized animals, and animals sacrificed during the experiment, it seemed desirable to undertake a study where the intact animal served as his own control and where hormone excretion studies were followed directly throughout the post-radiation period. Seventeen ketosteroid analyses were best adapted to such a study. Seventeen ketosteroids have been shown to rise for a short period and then fall with a final reversion of excretion to normal levels in response to trauma and disease (Forbes, *et al.*, 1947; Stevenson, Shenker and Browne, 1944; Cope, Nathanson, Rourke and Wilson, 1943). This hormonal excretion pattern is observed in the adaptation of the organism to stress as described by Selye (1946). The sources of this steroid are both the testes and the cortex in the male and the cortex alone in the female (Fraser, Albright, *et al.*, 1941).

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[1] The opinions are those of the author and are not to be construed as reflecting the views of the Navy Department.

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Therefore, this test would give an indication of at least one aspect of adrenal cortical function.

METHODS

Eleven dogs, eight males and three females, weighing from 9 to 19 kg. were used in the course of the experiment. These dogs were maintained on Purina dog chow and water *ad libitum*.

The dogs were irradiated in plywood cages, 26 cm. wide, which had been constructed so that the maxmum intensity of the angular beam of the X-ray was located at the horizontal mid-point of the dog. The cages were constructed to conform to a segment the arc of which was one meter (Chapman,

TABLE 1. NORMAL 17 KETOSTEROID EXCRETION VALUES IN MGM/24 HOURS

Dog	Sex	Wt./kg.	No. of determinations	Mean	Standard deviation	Range
49	F	9.3	12	1.05	0.59	0.23–2.00
47	F	12.4	13	1.65	1.41	0.39–4.86
37	F	11	11	0.85	0.58	0.23–2.40
21	M	12	10	1.35	0.65	0.73–2.63
32	M	12.2	13	1.65	1.28	0.51–5.14
50	M	10.4	10	0.75	0.38	0.22–1.34
27	M	16	30	1.82	0.79	0.42–2.95
23	M	16	20	1.85	1.09	1.75–5.43
6	M	17	22	3.06	0.63	0.65–5.19
1	M	14	12	2.04	1.30	0.77–3.44
40	M	19	39	1.69	0.86	0.43–4.89

et al., 1948). The radiation factors were: 1,000 KV, 3 ma, with an average intensity of 25.5 r/minute. The depth dosage using a pressedwood phantom of uniform density was 61 percent at 10 cm. and 13 percent at 25 cm. Dogs were radiated in two groups. Group one was composed of five male mongrels. Group two was composed of three male and three female purebred beagles as it was felt that this would render the biological response more uniform. A dose of 150 r was delivered to one side; the animals were turned around and 150 r was delivered to the other side for a total of 300 r. This had been determined as a probable minimal lethal dose at another laboratory (Boche, 1948).

Urine samples were collected in metabolism cages on a 24-hour basis. The washed neutral ether extract was then assayed for 17 ketosteroid content by the Zimmerman reaction as modified by Callow (1938) and Talbot (1940). Anhydrous aldehyde-free alcohol, purified 2 per cent m-dinitrobenzene, and 2.5 *N* alcholic potassium hydroxide were used. The reaction was developed for one hour at 25°C ± 0.2°C in darkness. The optical density of the colorimetric reaction was measured in an Evelyn photoelectric colorimeter using a filter having a maximum transmission at 520 mμ. The colorimeter was calibrated with known concentrations of crystalline dehydroisoandrosterone.

The first group of dogs was subjected to the minor stresses of being bled small amounts and of being transported in irradiation cages to the X-ray tube. They also received a dosage of 15 mgm. and 25 mgm. of purified adrenocorticotrophic hormone subcutaneously in an attempt to study adrenal reserve. These dosages were administered on different days three weeks before and ten days following radiation.

RESULTS

The normal values are recorded in table 1 as the number of determinations, the range, and the standard deviation of pre-irradiation values. These values agree with those reported elsewhere in the literature (Pashkis, Cantarow, *et al.*, 1943). These authors report no sexual difference. In the five dogs comprising the first group, minor stresses and a single dosage of adrenocorticotrophic hormone produced no significant change in the 24-hour 17 ketosteroid excretion.

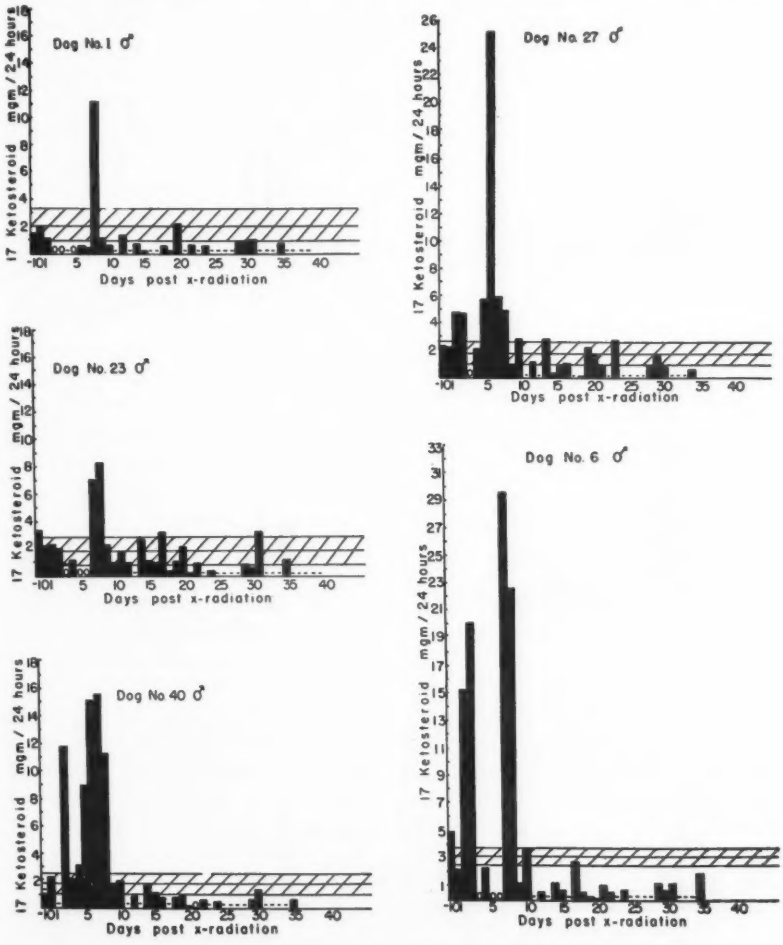

FIG. 1.—Seventeen ketosteroid excretion in X-radiated dogs. The ordinates represent steroid values in mgm. per twenty four hours. The asbcissas represent time in days. The shaded areas represent one standard deviation above and below the mean of normal determinations. O indicates no steroid was found in assay. —indicates no assay was made.

As may be observed in figures 1 and 2, eight animals 23, 1, 49, 47, 37, 21, 32, and 50 responded to radiation with an initial increase in 17 ketosteroid excretion which occurred between the fifth to twelfth day following radiation. The increase in all cases was greater than three times the standard deviation and greatly exceeded the normal range. The increased excretion was followed immediately by a fall to normal or subnormal levels and showed a definite trend to the normal range by the thirtieth to fortieth day.

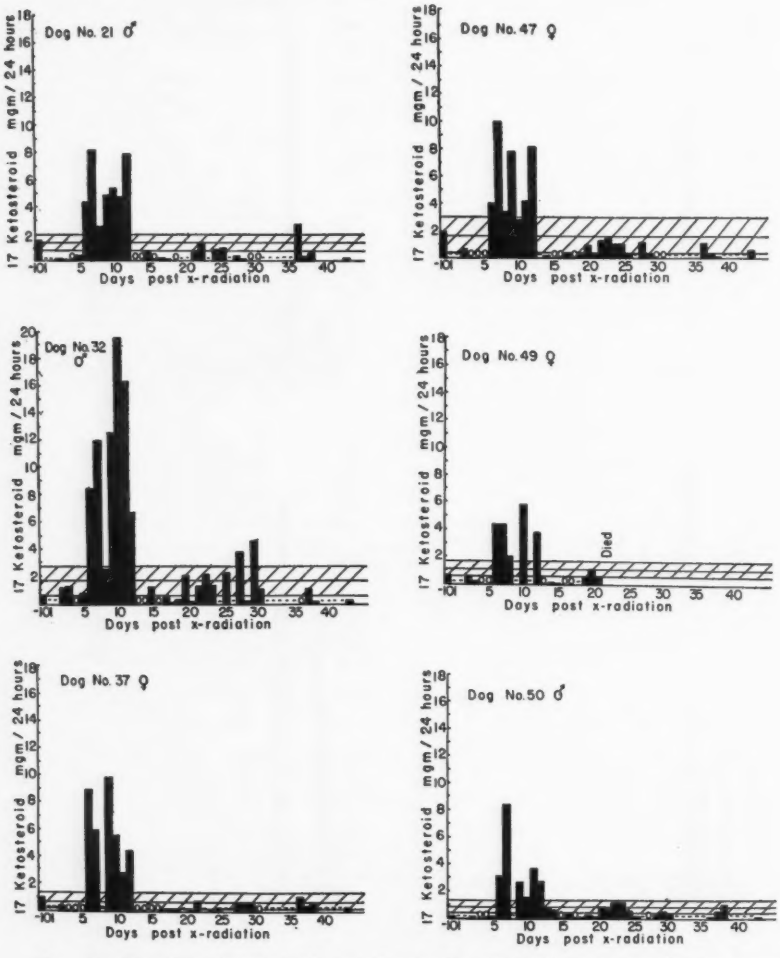

FIG. 2.—Seventeen ketosteroid excretion in X-radiated dogs. The ordinates represent steroid values in mgm. per twenty four hours. The abscissas represent time in days. The shaded areas represent one standard deviation above and below the mean of normal determinations. O indicates no steroid was found in assay. —indicates no assay was made.

In the first group, three male dogs 27, 17, 40 showed a significant rise in 17 ketosteroid excretion on the first and/or the second day following radiation. This was not observed in the second group.

No animals died during the course of the first experiment. One animal, 49, of the second group died on the twenty-first day following radiation. Her rise in 17 ketosteroid excretion although present was never marked.

DISCUSSION

Seventeen ketosteroid urinary excretion values increased in all dogs at some time between the fifth and twelfth day following radiation and then fell to normal or subnormal levels showing a final trend in steroid excretion to normal ranges by the thirtieth to fortieth day. This may be interpreted to indicate an initial period of increased adrenal cortical and/or testicular activity (Fraser, Forbes, Albright, 1941) in response to radiation. This pattern of response agrees with that observed in many cases of stress due to trauma and disease (Cope, *et al.*, 1943; Forbes, *et al.*, 1947; Stevenson, Schenker and Browne, 1944). The time between the stress and the appearance of the rise in 17 ketosteroids is longer in this group of animals than is reported for other forms of stress. Indirect evidence is available in the literature to confirm this delay as peculiar to irradiation. Schwartz (1949) found that several patients exhibited an increase in 11 oxycorticosteroids which occurred on the sixth day following radiation. Patt (1947) found the increase in adrenal cholesterol following LD_{50} and higher doses of radiation occurred on the sixth to seventh day. X-radiation has recently been reported to produce an initial immediate increase in 17 ketosteroid excretion in patients treated for rheumatoid arthritis. The excretion then fell to levels below the pre-irradiation levels which had been higher than the normals for healthy men and women (Davison, Loets, Kuzell, 1949).

Our findings at this one dosage range appear to be significant due to the uniformity of the excretion pattern. They agree with previous investigations that have illustrated an altered adrenal cortical function in response to X-radiation. The findings would also indicate that irradiation may produce an adaptation response similar to that seen following other stimuli. Assay of other adrenal cortical hormone excretion patterns as well as 17 ketosteroids at different X-ray dose levels will be necessary to establish this concept.

SUMMARY

The urinary excretion pattern of 17 ketosteroids was assayed in 11 dogs following exposure to a minimal lethal dose of total body X-radiation. All animals exhibited a significant increase in steroid excretion at some time between the fifth to twelfth day following radiation. These values then fell to normal or subnormal levels with excretion showing a trend to normal levels by the thirtieth to fortieth

day. This excretion pattern is interpreted to indicate an alteration of adrenal cortical and/or testicular activity in these animals at this dose range.

The hormonal excretion pattern is similar to that reported as occuring in the organisms adaptation to other stimuli. Further studies are necessary to establish this concept.

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THE EFFECTS OF THYROXIN AND THIOURACIL ON THE TIME OF APPEARANCE OF OSSIFICATION CENTERS OF RAT FETUSES

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INTRODUCTION

THE SECRETIONS of the thyroid gland tend to accelerate skeletal ageing while lack of the secretions tends to retard this ageing (Silberberg and Silbergerg, 1943, and others). The time of appearance of secondary ossification centers, a stage of skeletal ageing, seems to agree with this concept, for such centers are accelerated in their appearance in rats injected with thyroxin (Noback, Barnett and Kupperman, 1949) and delayed in their appearance in thiouracil-injected rats (Noback, Barnett and Kupperman, 1949), in human cretins (Means, 1937; Caffey, 1945; Albright, 1947; and others), and in thyroidectomized rats (Scow, Becks, Simpson, Asling and Evans 1948).

The purpose of this paper is to consider the relation of the thyroid gland to the time of appearance of primary ossification centers of membrane and cartilage bones (which only appear during prenatal life) in rat fetuses.

MATERIAL AND METHODS

Young adult female albino rats (Sprague-Dawley strain) were mated with males of the same strain. When sperm were identified in the morning vaginal smear or when a vaginal plug was found, the animal was considered to be pregnant as of that morning (Blandau, Boling and Young, 1939).

The experiments were divided into (1) a thyroxin series and (2) a thiouracil series. The distribution of the animals, and the dosage of drugs, in these experiments, is noted in Table 1.

After sacrificing the mothers, the fetuses were fixed in 95% alcohol, cleared and the osseous skeletons stained with alizarin red by the technique of Noback and Noback (1944). An ossification center of a bone was noted as present when stained by alizarin red. Several fetuses from each litter were prepared for observations not in the scope of this paper and hence not included in the number of fetuses observed as noted in Table 1. The thyroid glands of the mothers and the fetuses were fixed in 10% formalin.

The ossification centers examined are among the first to appear in the

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TABLE 1. SUMMARY OF NUMBER OF ANIMALS USED

Experiment	No. of mothers	No. of fetuses* examined for ossification centers	Total No.* of fetuses
Thyroxin Series			
Group I— (animals sacrificed at 15 days of pregnancy)			
Control animals:	4	24	32
Experimental animals: Mothers injected daily with 1 mg. Thyroxin[2] from 9th to 15th day of pregnancy.	5	26	37
Group II— (animals sacrificed at 16 days of pregnancy)			
Control animals:	8	53	74
Experimental animals: (a) Mothers injected daily with 1 mg. Thyroxin from 10th day through 16th day of pregnancy.	4	22	38
(b) Mothers injected daily with 1 mg. of Thyroxin from 6th to 16th day of pregnancy.	4	24	39
(c) Mothers injected daily with 0.1 mg. of Thyroxin from 3rd to 16th day of pregnancy.	3	33	33
Thiouracil Series			
Control animals: same as in thyroxin series group II.	8	53	74
Experimental animals: Mothers fed with 0.2–0.4% thiouracil[3] from first day of pregnancy until sacrificed.			
(a) Animals sacrificed at 16th day of pregnancy.	10	62	87
(b) Animals sacrificed at 17th day of pregnancy.	4	22	33

* Not all fetuses were examined for ossification centers as several fetuses in each litter were saved for observations not in scope of this paper.

fetus. The degree of development of each center was graded according to (1) a bone index scale and (2) a count of the number of ossified ribs. The bone index is an arbitrary and subjective scale which was devised in an attempt to give some reasonable numerical expression, from a low of 1.0 to a high of 4.0, to the developmental status of the bones observed. The

[2] The crystalline thyroxin was supplied through the generosity of Dr. C. H. Mann of E. R. Squibb and Sons.

[3] The thiouracil (2-thio-6-oxypyrimidine) was supplied through the generosity of Dr. S. N. Hardy and Dr. M. Lockhart of the Lederle Laboratories.

average bone index for each center was computed by averaging all the bone indices of each center for all the animals in each group of experiments. The standard deviation and probable error were calculated for the average bone index of each center. Then the differences between the control bone indices were compared to see if they were statistically significant. The degree of statistical significance of the experimental animals compared to the control animals is tabulated in Table 2.

OBSERVATIONS

Thyroxin Series, Table 1, Figures 1 and 2

The 15 day-old fetuses of the control animals and thyroxin-injected animals had no ossification centers.

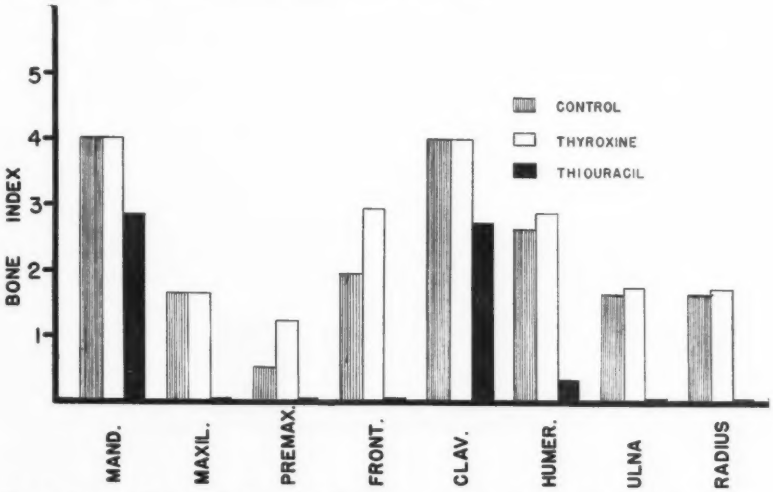

FIG. 1. Bone indices of 16 day-old fetuses from control animals and of those mothers which were injected with thyroxine from the 6th day of pregnancy or fed thiouracil.

The 16 day-old fetuses of the control animals and both series of thyroxin-injected animals had ossification centers for the mandible, maxilla, premaxilla, frontal bone, clavicle, humerous, ulna, radius and ribs. No statistically significant differences were noted between the control animals and any of the series of thyroxin-injected animals, as regards the bone indices and number of ribs.

Thiouracil Series, Table 1, Figures 1 and 2

In this series the 16 day-old control animals are the same animals used in the thyroxin experiment.

The number of ossification centers in the 16 day-old fetuses of females fed the diet containing thiouracil was less than in the control fetuses. Only the mandible, clavicle, humerus, and ribs had ossified,

and the degree of development of their ossification center was greatly reduced as indicated by the bone indices in Figure 1. The number of ossified ribs was 2.1 $\pm$0.5 in the experimental animals compared to 9.6$\pm$0.2 in the control animals. In this experiment there are statistically significant differences between the fetuses of the control animals and of the thiouracil-fed animals as regards the bone indices and number of ossified ribs (Table 2).

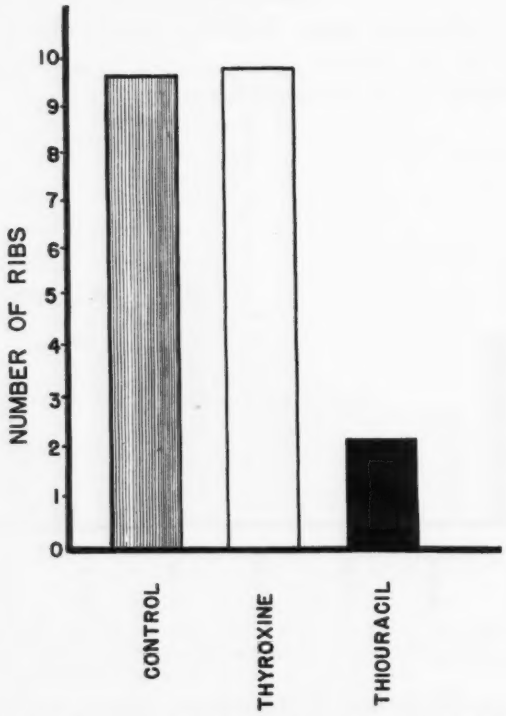

FIG. 2. Number of ribs present in 16 day-old fetuses of animals noted in Fig. 1.

The 17 day-old fetuses of mothers fed thiouracil throughout pregnancy were examined to see if the centers which failed to appear (maxilla, premaxilla, frontal bone, radius and ulna) in the 16 day-old fetuses of thiouracil-fed mothers had appeared. In these 17 day-old fetuses, all of the centers listed above had appeared.

Thyroid Glands of Animals of the Thyroxin and the Thiouracil Series

The maternal thyroid glands showed histological evidence that the thyroxin and the thiouracil were pharmacologically active. The thyroid glands of thyroxin-injected mothers had atrophic acinar cells

TABLE 2. DEGREE OF SIGNIFICANCE (xP.E.)* OF DATA OBTAINED FROM FETUSES OF MOTHERS SACRIFICED ON THE 16TH DAY OF PREGNANCY COMPARING CONTROL AND EXPERIMENTAL ANIMALS

Bone	Degree of significance (xP.E.)* of data obtained from fetuses of mothers sacrificed on the 16th day of pregnancy		
	Mothers injected with thyroxine from the 10th day of pregnancy	Mothers injected with thyroxine from the 6th day of pregnancy	Mothers fed diet containing thiouracil
Mandible	0.0	0.0	8.6
Maxilla	0.9	0.0	16.0
Premaxilla	1.4	2.3	5.0
Frontal Bone	1.0	2.3	9.5
Clavicle	0.0	0.0	9.3
Humerus	1.4	1.4	10.0
Ulna	0.0	0.7	16.0
Radius	0.0	0.7	16.0
No. of Ribs	0.3	1.4	15.0

* A xP.E. greater than 3.0 denotes statistical significance.

and acinar lumina filled with colloid. The hyperplastic glands of thiouracil-fed mothers had hyperplastic acinar cells, most acinar lumina with no colloid, and a few lumina with traces of colloid.

The drugs used apparently had no effect on the fetal thyroid glands, since the differentiating follicles of the fetuses of control, thyroixin-injected and thiouracil-fed animals were microscopically similar.

DISCUSSION

On the basis of the above data it may be concluded that for the dosage of thyroxin injected into the pregnant rats, thyroxin does not significantly influence the time of appearance of the fetal ossification centers under observation. These thyroxin results differ from those of Noback, Barnett and Kupperman (1949) who noted that daily injections of thyroxin into postnatal rats accelerated the appearance of ossification centers appearing 8 to 10 days after birth. The length of time our animals were injected with thyroxin equalled or exceeded the length of time that Noback, Barnett and Kupperman (1949) injected their rats with thyroxin. Thus on the basis of their data the durations of our experiments were sufficient. There are several possible explanations for the difference between the effects of thyroxin on the time of appearance of prenatally appearing centers and postnatally appearing centers. Since evidence in the literature indicates that the thyroid hormone may pass through the placenta of the guinea pig (whose placenta is similar to the rat placenta), and dog (Halstead, 1886; Ukita, 1919; Döderlein, 1928), the differences may not be explained by the lack of any of the injected hormone in the fetus. An explanation may be that the amount of hormone passing through the barrier is insufficient to accelerate the appearance of the ossification centers. It may also be that the fetus is under maximal thyroid sti-

mulation from the mother in the normal pregnant rat and is refractory to further thyroxin stimulation.

As regards the thiouracil experiments, it may be concluded that thiouracil delays the time of appearance of the fetal ossification centers examined. Since thiouracil inhibits the production of endogenous thyroid hormone (Astwood, 1949), it may be inferred that the delay in the appearance of the centers is probably due to thyroid hormone insufficiency. Compensatory fetal thyroid activity is inhibited since thiouracil passes through rat placental barriers (Freisleben and Kjervuld-Jansen, 1946). This is similar to the observations on the delay of the appearance of secondary ossification centers in human cretins (Albright, 1947; Means, 1937; Caffey, 1947, and others), in thyroidectomized rats (Scow *et al.*, 1948), and in thiouracil-injected rats (Noback, Barnett and Kupperman, 1949).

Our data may indicate that the maternal thyroid gland secretions have a role in the differentiation of the ossification centers studied in the 16 day-old fetus. This is based on the assumption that the evidence in the literature is conclusive in indicating that the fetal rat thyroid gland does not become active until the 17th to 19th day of pregnancy. Colloid in the thyroid follicles is first seen in 17 day fetal rats[4] (Hall and Kaan, 1942). No follicular colloid is present in any of the 16 day-old rats in our series. The 19 day-old fetal rat[4] thyroid gland shows physiological activity as measured by the effects of the injection of these glands on tadpole metamorphosis (Hall and Kaan, 1942). Maternally-injected radio-active iodine is first concentrated in the thyroid gland of the 18 to 19 day-old rat fetus (Gorbman and Evans, 1941). If the rat fetal thyroid gland does not secrete thyroid hormone by the 16th day of pregnancy, then the fact that the centers of ossification were delayed in their appearance in the 16 day-old fetuses of mothers injected with thiouracil suggests that the maternal thyroid hormone has a role in the time of appearance of the ossification centers studied.

These results indicate that the thyroid gland has a role in the differentiation of membrane bones (frontal bone, etc.) and of primary endochondral bones (humerus, etc.).

SUMMARY

The time of appearance of the ossification centers studied was not significantly altered in the 16 day-old rat fetuses the mothers of which were injected daily with 1 mg. of crystalline thyroxin from either the 6th or the 10th day after pregnancy.

The time of appearance of the same ossification centers was de-

[4] To make the fetal ages comparable we are adding one day to Hall and Kaan's (1942) ages since these authors calculated fetal age commencing one day after sperm were observed in the vaginal smears while our calculations commenced on the day sperms were observed in the vaginal smears.

finitely delayed in the 16-day-old rat fetuses the mothers of which were fed on a diet containing 0.2 to 0.4% thiouracil from date of pregnancy.

On the basis of these results it may be concluded that the thyroid gland has a role in influencing the time of appearance (differentiation) of the ossification centers of membrane bones and primary endochondral bones. Furthermore, it is suggested that thyroid hormone from the maternal thyroid gland may be essential for normal differentiation of the observed ossification centers appearing in 16 day-old rat fetuses.

ACKNOWLEDGMENTS

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THE METABOLIC RATE AND THYROID SIZE OF CHICKS FROM DAMS WITH ALTERED METABOLISM[1]

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THE EFFECT of the thyroid hormone and synthetic thyroxine or thyroprotein and goiterogenic substances, such as thiouracil, on the metabolic rate and thyroid size of experimental animals has been well established. Relatively little information is available, however, on the effect of hyperthyroidism or hypothyroidism of the dam on the metabolic rate of her progeny.

Hens fed a ration containing thyroprotein have been shown to produce chicks with enlarged or goiterous thyroid glands (Wheeler and Hoffman, 1948a, and McCartney and Shaffner, 1949). Chicks with goiterous thyroids have also been shown by Andrews and Schnetzler (1945) to be produced by females fed thiouracil in the ration.

Since the chicks from thryoprotein-fed hens had a prolonged incubation period, a significantly greater thyroid size, and a lowered heart rate, Wheeler and Hoffman (1948a) concluded that they were hypothyroid. Assay of chick thyroidal activity, as determined by the closed-vessel technique of Smith **et al.** (1947), led McCartney and Shaffner (1949) to believe that the metabolism of chicks produced by both thyroprotein-fed and thiouracil-fed females is lower than that of normal chicks, suggesting a reduced metabolic rate and a functional hypothyroid condition.

The present experiment was undertaken to further investigate these unexpected observations and to study the mode of action of the compounds and the post-natal duration of the effects.

METHOD

On September 1, 1948, sixty New Hampshire pullets were selected and placed in individual laying cages. After four weeks, these females were divided at random into three groups of twenty birds each. Thyroprotein[3] was added to the ration of one group at a level of 0.022 per cent and thioura-

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[3] The thyroprotein used in this experiment, called Protamone, was kindly supplied by Dr. W. R. Graham, Jr., of the Cerophyl Laboratories, Inc., Kansas City, Missouri.

cil[4] to the ration of a second group at a level of 0.1 per cent. The third group served as controls.

The resting metabolism of these females was determined at 4-week intervals by means of the indirect calorimetry method, employing a closed circuit type of respiratory apparatus. All metabolism tests were run at night between 6 P.M. and 12 P.M. after the birds had been fasted for approximately twelve hours. All measurements were recorded on the basis of cubic centimeters of oxygen consumed per kilogram of body weight per hour, corrected to standard temperature and pressure.

In order to measure the thyroidal activity of the day-old chicks produced by the different groups of females, the oxygen consumption of groups of 18 chicks each, maintained at 32° to 35°C., was measured. The method used to obtain these determinations was the same as that employed to estimate the resting metabolism of their dams. A series of eight observations were made on chicks produced by each of the three different groups of females over a period of 12 weeks.

To determine the rate at which the chick's thyroid gland size was affected by feeding its dam thiouracil or thyroprotein, groups of day-old chicks (10 per group) were sacrificed at weekly intervals during the treatment period and their thyroid glands weighed. These observations were made beginning with the first hatch after the initiation of treatment and continued for the next ten successive weekly hatches. All thyroids were freshly dissected and weighed on a Roller-Smith balance to the nearest 0.1 milligram.

To study the effect of these treatments on the post-natal changes in thyroid size, chicks produced by the three groups of females were reared in electric battery-brooders with raised screen floors and were fed a conventional all-mash ration. Samples of 10 chicks at day-old and at 7, 14, and 21 days of age, respectively, were sacrificed, the thyroid glands removed and weighed to the nearest 0.1 milligram.

In studying the effect of exogenous thyroxine on the thyroid size of the chick, eggs from control females were injected with aqueous solutions of thyroxine or thyroprotein by puncturing a small hole in the shell over the air cell and applying the solution by means of a tuberculin syringe on the inner shell membrane. Following the injections, the holes were sealed with paraffin wax and the eggs placed in the incubator. Thyroxine was injected at levels of 6, 12, 20, and 40 micrograms and thyroprotein at levels equivalent to 9, 12, 20, and 40 micrograms of d,l-thyroxine. Similarly, eggs produced by thyroprotein-fed females were also injected with thyroxine, at levels of 2, 4, 6, 8, and 10 micrograms.

Since iodized casein preparations have been shown to contain from 7.7 to 8.7 per cent iodine (Harrington and Pitt Rivers, 1939), it was thought that possibly excess amounts of iodine may have been transmitted to the eggs produced by the thyroprotein-fed females, producing chicks with enlarged thyroids. Therefore, iodine in the form of KI was injected into control eggs at levels of 4, 6, 8, 10, 20, and 40 micrograms, respectively. Day-old chicks hatched from the injected eggs were sacrificed and the thyroid glands weighed to the nearest 0.1 milligram. Approximately 500 eggs were used in the thyroxine, thyroprotein, and KI injection studies.

[4] The thiouracil (Deravet) was supplied through the courtesy of the Lederle Laboratories, Pearl River, New York.

RESULTS

The average oxygen consumption of the control females and of the females receiving thyroprotein or thiouracil in their ration is shown in Table 1. These data indicate that the addition of 0.022 per cent thyroprotein to the ration brought about a small but highly significant increase in the rate of resting metabolism. After 12 weeks of treatment the average rate of resting metabolism of these females was about 12 per cent more than that of the control females. Feeding 0.1 per cent thiouracil in the ration caused a marked decrease in the rate of metabolism. The average rate of resting metabolism of these females was approximately 24 per cent less than that of the controls. This difference in oxygen consumption of 204 ± 30 cc. per kilogram of body weight per hour between the control females and the females fed thiouracil for 12 weeks is highly significant statistically. These results provide sufficient evidence to indicate that mild hyperthyroidism and hypothyroidism were experimentally induced in the females receiving thyroprotein and thiouracil, respectively, in their ration.

TABLE 1. RESTING METABOLISM OF CONTROL FEMALES AND FEMALES FED THYROPROTEIN OR THIOURACIL

Weeks of treatment	Cc. O_2/kilo/hour*		
	Control	Thyroprotein	Thiouracil
0	934 ± 39	—	—
4	930 ± 27	982 ± 47	837 ± 31†
8	798 ± 20	1012 ± 35†	716 ± 24†
12	843 ± 25	943 ± 32†	639 ± 18†

* Cubic centimeters of oxygen consumed per kilogram of body weight per hour.
† The difference between these values and the control is highly significant.

The average oxygen consumption, in milliliters per kilogram of body weight per hour was 1981 ± 74 cc. in chicks from the control females, 1660 ± 115 cc. in chicks from the females receiving thyroprotein, and 1644 ± 90 cc. in chicks from the thiouracil-fed females. The chick-metabolism tests were made during the last half of the 12-week treatment period of their dams. The chicks produced by the thyroprotein-fed and the thiouracil-fed females consumed 16.2 and 16.5 per cent, respectively, less oxygen than the control chicks. In each case, the decrease in oxygen consumption of the chicks from the treated dams is statistically significant.

In Figure 1 are presented results showing the progressive change in chick thyroid size following initiation of treatment to their dams. The results of this study indicate that the thyroid glands of the chicks produced by the thiouracil-fed females had not attained their maximum size after 11 weeks of treatment, while the thyroids of the chicks produced by the thyroprotein-fed females had apparently reached their greatest size (about 11.0 milligrams) after 8 weeks of treatment.

The lines shown in Figure 2 represent the change occurring in the

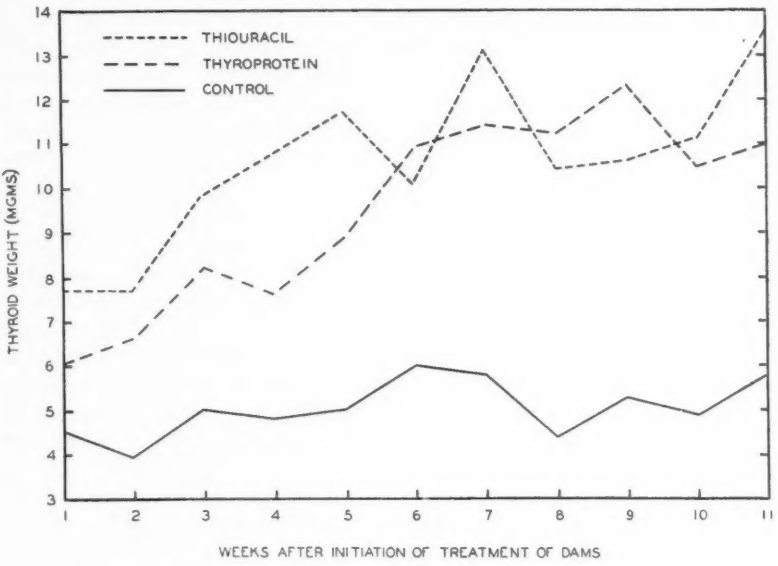

FIG. 1.—Thyroid weight of day-old chicks from control dams and dams fed thiouracil or thyroprotein.

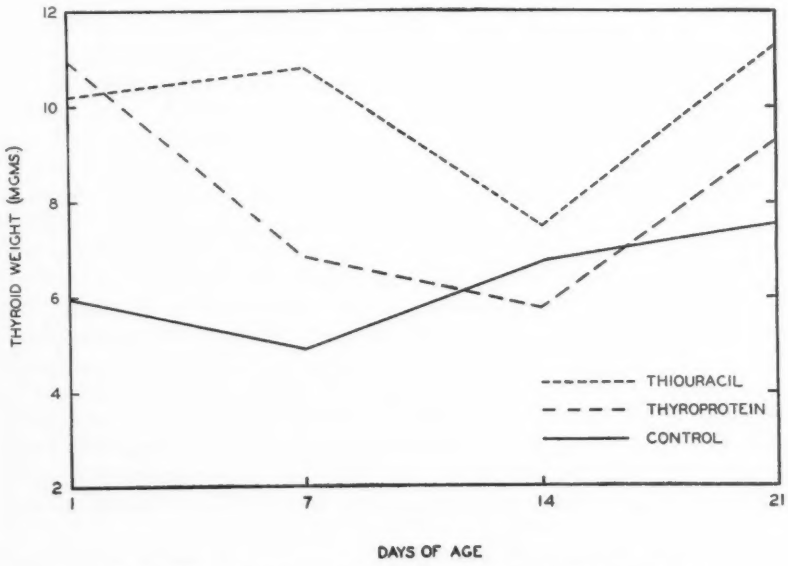

FIG. 2.—Change in thyroid weight of chicks produced by control dams fed thiouracil or thyroprotein (day-old to 21 days of age).

thyroid weight from day-old to three weeks of age of chicks produced by the three groups of females. The thyroid weights of the chicks from the thyroprotein-fed females decreased during the first week, while those of the chicks from the thiouracil-fed females increased somewhat. By the end of the second week the thyroid glands of the chicks from the treated dams were comparable in size with those of the controls. The thyroids of the three groups of chicks increased slightly during the third week of the post-hatching period.

McCartney and Shaffner (1949) have previously shown that chicks with enlarged thyroids were produced by females fed a ration containing thyroprotein or thiouracil, while a decrease in thyroid size resulted when thyroxine was injected into control eggs. The thyroid glands of the chicks hatched from eggs injected with 6, 12, 20, and 40 micrograms, respectively, of d,l-thyroxine were consistently smaller than the thyroids of normal chicks. Preliminary studies with thyroprotein-injected eggs (9 and 12 micrograms) were too limited to determine its effects on thyroid size. However, subsequent studies revealed that thyroprotein injected into control eggs at levels equivalent to 20 and 40 micrograms of d,l-thyroxine had no effect upon chick thyroid size.

Since chicks with goitrous thyroids are produced by thyroprotein-fed females, eggs laid by these females were injected with thyroxine at levels of 2, 4, 6, 8, and 10 micrograms. The results of these thyroxine-injection studies revealed that approximately 6 micrograms of exogenous thyroxine is necessary to affect thyroids of normal size in chicks produced by thyroprotein-fed females.

The thyroid glands of chicks hatched from control eggs injected with KI at levels as high as 40 micrograms were comparable in size with the thyroids of normal chicks.

DISCUSSION

The results obtained on the rate of resting metabolism indicate that mild hyperthyroidism and hypothyroidism were experimentally induced in the females fed 0.022 per cent thyroprotein and 0.1 per cent thiouracil, respectively, in their ration. The rate of oxygen consumption of the thyroprotein-fed females, after 12 weeks of treatment, was 12 per cent more than that of the control females. This increase in metabolism is comparable with the changes in metabolism obtained in other animals by feeding thyroprotein. The decrease of approximately 30 per cent in the resting metabolism of the thiouracil-fed female chickens during a period of 12 weeks is somewhat less than that reported by Shaffner and Andrews (1948) for male chickens during a similar period of time, indicating that there may be a sex difference in response to thiouracil.

Day-old chicks produced by each of the two groups of treated females required about 16 per cent less oxygen than the control chicks. Since these resting metabolism results are in fairly close agree-

ment with the survival-time results reported by McCartney and Shaffner (1949), it seems that there is little doubt that the chicks produced by both the hyperthyroid and the hypothyroid dams are themselves hypothyroid.

According to Wheeler and Hoffman (1948b), the thyroid enlargement of chicks from thyroprotein-fed hens is maximum about 14 days after the initiation of treatment. These results are not in accord with the findings reported herein, since in this experiment chick thyroid enlargement did not reach a maximum (greatest size of approximately 11.0 milligrams) until 8 weeks after the females were put on the experimental ration. If chicks with enlarged thyroids are the result of a decreased secretion of the maternal thyroid hormone in females fed thyroprotein, this indicates that the maximum reduction in deposition of maternal thyroxine was not reached until several weeks after the exogenous thyroxine was administered in the ration. However, chicks with goitrous thyroids occurred concurrently with the addition of thyroprotein to the ration of their dams, the thyroids being significantly larger within one week from the beginning of treatment.

The thyroid glands of the chicks produced by the thiouracil-fed females were still increasing in size after eleven weeks of treatment. This progressive increase in thyroid size would indicate that more thiouracil and/or less thyroxine was transmitted from the female to her eggs as the treatment advanced. Since the resting metabolism of these females decreased progressively during the experimental period, it is apparent that a relationship exists between the degree of hypothyroidism of the female and the amount of thyroid enlargement of her chicks.

Since the thyroid glands of the chicks from thiouracil-fed dams continued to enlarge during the first week after hatching, it would appear that thiouracil was stored in the chick's yolk sac. The thyroid weight of the chicks produced by the thyroprotein-fed females decreased during the first week, suggesting that there is little or no carry over in the yolk sac of the factor or factors causing thyroid enlargement in these chicks.

Thyroprotein injected into control eggs at levels as high as 40 micrograms of d,l-thyroxine had no effect on chick thyroid size, indicating that the thyroprotein cannot be assimilated or utilized by the developing chick embryo. It seems logical to assume that the thyroxine in a solution of thyroprotein should pass through the shell membrane as readily as a solution of synthetic thyroxine. Booker and Sturkie (1949) injected thyroprotein into the albumen of incubating eggs at a level of 33 gamma (equivalent to 1 gamma of d,l-thyroxine) and obtained a slight decrease in thyroid size. However, in view of the limited number of observations cited, the difference between the thyroid size of the chicks from the control eggs and the injected eggs could have been easily due to sex. Males have significantly smaller thyroids than females (Aberle and Landauer, 1935).

The injections of thyroxine into eggs laid by the thyroprotein-fed females consistently reduced thyroid size, indicating that there was actually a deficiency of thyroxine in these eggs. Approximately 6 micrograms of exogenous thyroxine was necessary to affect thyroid glands of normal size in chicks from eggs produced by females receiving 0.022 per cent thyroprotein in the ration.

The results of the KI injections indicate that excess iodine transmitted from the dam to her eggs was not responsible for causing chicks with enlarged thyroids. Since a laying hen consumes about one-quarter of a pound of feed daily, it was assumed that the daily intake of iodine in thyroprotein was somewhere within the limits of 300 and 400 micrograms. Since only a small per cent of the total iodine intake of the thyroprotein-fed females could possibly be transmitted to their eggs, it seems probable that 40 micrograms of injected KI would be comparable to the maximum amount of iodine that could be transmitted from these dams to their eggs. Albert et al. (1946) injected groups of day-old cockerels (10 to 20 per group) with solutions of KI and has shown that KI injected at levels as high as 10 milligrams per chick had no effect on thyroid size.

SUMMARY

Three groups of New Hampshire females, 20 per group, were each maintained on a control ration, a ration containing 0.022 per cent thyroprotein, and a ration containing 0.1 per cent thiouracil, respectively. After 12 weeks the resting metabolism of the thyroprotein-fed and the thiouracil-fed females was 12 per cent more and 24 per cent less, respectively, than that of the control females.

Day-old chicks produced by each of the two groups of treated females required about 16 per cent less oxygen than the control chicks. The results of the metabolism studies indicate that chicks produced by both hyperthyroid and hypothyroid females are themselves hypothyroid.

About 6 micrograms of exogenous thyroxine were necessary to affect thyroids of normal size in chicks hatched from eggs laid by thyroprotein-fed females. Aqueous solutions of thyroprotein or KI injected into control eggs had no effect on chick thyroid size.

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THE EFFECTS OF STRESS, ADRENAL AND ADRENOCORTICOTROPHIC HORMONES ON THE CIRCULATING EOSINOPHILS OF MICE[1]

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THE EOSINOPHIL cell of the blood was first described over 100 years ago (Wharton Jones, 1846), and since then has been the subject of many morphological, cytological, and physiological investigations. However, despite the vast array of publications and reviews, the actual function of this cell in health or disease still eludes the investigator (Kirk, 1942; Bethell, Sturgis, Rundles and Meyers, 1946). A number of comprehensive studies have been made during the last 17 years but most of the investigations were carried out either in man or the guinea pig. Work in other animals seems to have been chiefly of a tabulative and descriptive nature (Scarborough, 1930).

In man, an increase in the number of circulating eosinophils has been correlated with allergy, parasitic infection, skin diseases, and a number of other disorders. In the guinea pig the eosinophils are associated with foreign protein reactions such as anaphylaxis and immunity. (For extensive references and reviews see: Schwarz, 1914; Ringeon, 1938; Rud, 1947; and Samter, 1949.)

The factors involved in decreasing the number of eosinophils seem to be alike in at least one respect, they all produce a stress. In 1939 Dalton and Selye pointed out in the rat that the eosinophils first decreased in number and then increased after various alarming stimuli. Selye (1947) later included this pattern of response in his concept of the general adaptation syndrome. More recently Hills, Forsham and Finch (1948) demonstrated that ACTH and Compound F produced a consistent decrease in the number of circulating eosinophils. They also reviewed the literature briefly and noted that eosinopenia follows various stress stimuli such as hemorrhage, cold, hemolysis, poisons,

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operative procedures, and a wide variety of infections. At the same time Forsham, Thorn, Prunty and Hills (1948) incorporated this decrease of eosinophils after ACTH injections into a clinical test to determine whether the adrenal cortex was functioning normally. These experiments indicate that a decrease in the number of circulating eosinophils is associated with an increased adrenal cortical activity.

The greatest handicap in working with eosinophils is the relatively small number of cells found in normal blood. Using the blood film method, large numbers of cells must be counted in order to determine accurately the percentage of eosinophils. However, in clinical work, this was overcome to some degree by Dunger who in 1910 reported a diluent for blood which stains the eosinophils and destroys the other blood cells. This was based upon the known fact that the eosinophil is the most resistant cell of the blood, especially to many hypotonic and acetone solutions. Since 1910 many workers have used the Dunger or similar procedures. Recently Rud (1947) improved this diluent and applied extensive statistical analyses to his techniques and results. He pointed out that the direct chamber method is superior to the blood film method when quantitative variation of the eosinophil is to be determined. Discombe (1946) agrees with this and states that the uncertainty of the hemocytometer count varies with the square root of the number of eosinophils counted. Randolph (1944) claimed that propylene glycol is superior to acetone as a diluent for human blood.

The authors are not aware of any specific eosinophil diluent devised for use in animal studies, although a number of vital dyes have been used to study the blood picture as a whole (Cunningham and Tompkins, 1938). Preliminary investigations in the mouse and rat had indicated that the eosinophils respond specifically and quantitatively to adrenal cortical compounds. It seemed advisable therefore to work out an eosinophil diluent which could be used in rodents. The following paper consists of a detailed account of the general techniques used for the determination of eosinophil cells in mice and rats, and the application of these techniques to a study of eosinophil numbers as influenced by various factors. A second paper is now being written which will describe a suggested procedure for determining quantitatively the amount of adrenal cortical hormone in various extracts and preparations.

MATERIALS AND METHODS

Two general techniques have been used in these studies. The first method is a slight modification of the standard clinical procedures (Lillie, 1948). Total white blood cell counts are obtained by diluting the blood in a 4% acetic acid solution. Differential counts were made from blood films prepared as follows: Thin smears are made on slides and fixed for 15 minutes in absolute methyl alcohol. The slides are then placed in a coplin jar and held

approximately 5 millimeters off the bottom of the jar to permit circulation of fluid beneath and between the slides. 40 cc. of diluent[3] is poured over the slides and then by use of a medicine dropper, 1½ cc. of concentrated stain[4] is mixed with the diluent. Care must be taken to avoid direct contact of the concentrated stain with the film side of the slide and to mix the concentrated stain as quickly as possible with the diluent. Occasional agitation is necessary during the staining process. The staining time varies from 2 to 8 minutes depending upon the density of stain desired and the type of blood used. The stain can be rapidly flooded out of the coplin jar with tap water, or the slides removed one at a time and rinsed quickly under the tap.

When the staining time is short, the eosinophil cells contain large reddish granules and very pale blue chromatin; and as the staining time is increased the chromatin stains more intensely. The neutrophil cytoplasm remains colorless and contains extremely minute granules; the lymphocyte cytoplasm stains a blue color.

The above procedure was found to have definite advantages over the standard methods for staining blood, especially mouse blood. The eosinophil granules were brought out much more distinctly. As many as 8 slides could be stained at once, and consistent results obtained from slide to slide. No precipitate forms on the slides.

Differential counts of at least 400 cells were made. To simplify the procedure we divided the white blood cells into 3 types:

1. Mononuclear cells, which include lymphocytes and monocytes.
2. Polymorphonuclear cells, which include neutrophils and all other myelogenous cells except eosinophils.
3. Eosinophil cells, which are cells containing numerous large reddish granules in the cytoplasm. The nuclei are usually bilobed or annular.

The absolute number of each type of cell was determined by multiplying the per cent of each cell in the differential count by the total number of blood cells.

However, it soon became evident that if 50 or more determinations of eosinophils were going to be made per day, that the procedure of total and differential counts took too much time. A survey was made of the various methods of determining eosinophils, but none was found applicable to the mouse or rat with any degree of accuracy. An experimental study was undertaken, therefore, to determine whether it was possible to obtain a diluting solution which would destroy the red blood cells and all the white blood cells except the eosinophils. Numerous water and mild alkaline solutions do this but the fragility of the eosinophils is increased so that any shaking of the diluting pipette causes them to disintegrate rapidly. Acetone was found to be more efficient in preventing the breakdown than any other substance used.

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[3] Diluent consists of:

680 cc. Distilled Water
120 cc. Acetone
200 cc Buffer at pH 7.3'
(McIlvaine's or Sorenson's)

[4] Concentrated Stain consists of:

1 gram powdered Wright's Blood Stain
1 gram powdered Giemsa's Blood Stain
75 cc. Diethylene Glycol
25 cc. Glycerine

In very dilute solutions (less than 5% in water) lysis of all cells occurred especially after shaking. As the concentration of the acetone was increased to 25%, more and more of the blood cells remained intact. A 15% acetone solution was found to give the best results in mice and rats. At this concentration the eosinophils remained intact with distinct cell membranes, while the other white blood cells formed hazy images in the background. The red cells were almost completely destroyed.

Phloxine was found to be a good stain for bringing out the eosinophil granules in the acetone solution. A small amount of detergent was added to aid in obtaining a better distribution of cells. Diethylene glycol was added to increase the viscosity of the solution and in some instances it was found to increase the total number of remaining eosinophils. The following formula was found most satisfactory in the mouse and rat:

10 cc. 0.1% Phloxine Solution
1 cc. Diethylene Glycol
4 drops 0.5% Alconox (Detergent)
30 cc. Distilled Water.
Just Before Using Add:
7 cc. Acetone

The eosinophils are recognized as follows: the granules within the cell stand out as distinct red bodies; they may be in a mass in the center of the cell, or scattered around the periphery. Usually the cell outline is seen only faintly, and the nucleus is completely colorless. The rat eosinophil is much larger and stains more deeply than the mouse eosinophil.

The procedure used to obtain blood from mice and rats is very important. Great variation in the number of eosinophils will be obtained for the same animal from one pipette to another unless a very highly standardized procedure is followed. For this reason, we include here in considerable detail the procedure we have found most satisfactory for obtaining a direct eosinophil count in mice. Slight modifications of the same procedure will give satisfactory results in rats.

Materials needed for a direct eosinophil determination:

WBC diluting pipettes and rubber aspirators
Fuchs' Rosenthal Counting Chambers, 0.2 mm depth
Microscope, preferably bifocal with 15× wide field oculars and 16 mm. objective.
Thin new razor blades for cutting tail
Cheese cloth for holding animals
Cleansing tissues
Eosinophil diluting fluid

Procedure for obtaining a blood sample in the mouse:

1. The mice are heated for approximately 10 minutes under a 100 watt lamp. This is best done in a battery jar containing about 1 inch of wood chips at the bottom. The mice should become active so that the blood circulation in the tail is stimulated.
2. A mouse is then wrapped loosely in cheesecloth with the tail projecting out, and held firmly in the palm of one hand. The tail is washed with soap and warm water.
3. With a sharp razor a small nick is made through the skin, then through one of the prominent blood vessels just beneath the skin. If properly

done, large drops of blood will quickly form over the cut, and the tail will later heal over without showing any deformity.

4. The first two drops of blood are discarded and the wound and surrounding area carefully wiped with a cleansing tissue to remove any debris and blood. Fresh blood is then steadily drawn into the WBC pipette as it oozes from the wound until it reaches the 0.5 mark on the pipette. Avoid taking blood that has been exposed to air for more than a few seconds, or which shows any clot formation.
5. The eosinophil diluent is quickly drawn into the pipette as the pipette is rotated between the fingers to facilitate mixing of blood and diluent. Fill to the 11 mark and shake gently by hand for 5 to 10 seconds. Fill the counting chamber immediately, discarding the first 3 drops and placing the 4th and 6th drop one on each side of the chamber.
6. Allow the counting chamber to stand for at least 3 minutes and carefully count the number of cells in each chamber. The number of cells in 16 squares (one side) multiplied by 6.25 equal the number of cells per cubic millimeter of blood.

The acetone prevents the breaking down of the white blood cells. Therefore, if the eosinophils appear broken and the granules to be spreading, add more acetone to the diluent. If the other white blood cells remain and hinder the recognition of the eosinophils, decrease the amount of acetone in the diluent. If the granules are not stained deeply enough, allow the chamber to stand for a few minutes longer before counting. If still lightly stained, decrease the acetone in the diluent. It is usually better to remake the eosinophil diluent every time it is used. We keep stock solutions on hand which can be mixed quickly, although on occasion acetone can be added to old diluent and satisfactory counts obtained.

It takes some experience and practice to make accurate determinations of the numbers of eosinophils by the direct method. It is to be expected that the cells in the hemocytometer chamber will be distributed in a Poisson fashion and this has been observed by numerous workers (Discombe, 1946; Rud, 1947). If the cells are distributed in this manner the variance should be equal to the mean number of cells counted. This was checked by making two duplicate counts from each of 60 pipettings covering a wide range of eosinophil concentrations. The value of χ^2 turned out to be 60.79 which for 60 degrees of freedom corresponds to a probability of .45, indicating satisfactory agreement of the data with the Poisson hypothesis.

However, there are errors other than those of the counting chamber in an estimation of eosinophils by the direct method. For the purpose of determining the over-all error, including that which might be due to variation in eosinophil concentrations in different drops of blood from the same animal, counts were made on four successive drops of blood from each of 20 animals. As was to be expected, the variance was greater than that of the counting chamber alone, but there was still proportionality between the variance and the mean number of cells counted, or between the standard deviation and the square root of the number counted. For this series, the ratio of the standard deviation to the mean number of cells counted averaged 1.70. If this figure is taken at face value, one can say that the standard deviation of a given cell count is about 1.7 times the square root of the actual number of cells enumerated.

It should be emphasized that the value of 1.7 was obtained from only

80 counts on 20 animals and that more data may require that this figure be adjusted. Improvements in technique probably would lower the error somewhat, although it could never be less than one times the square root of the number of cells counted. However, this provides an approximate estimate of the magnitude of error of a single count and shows that this is much less than that of ordinary differential counts (Rud, 1947). The error can be further reduced, if need be, by taking duplicate counts from the same animal.

Error of a greater magnitude seems to occur in the counts of blood from those animals which are not sufficiently active to produce good circulation of blood in their tails. This is particularly true if the tail has to be milked to obtain enough blood. Furthermore sick animals that do not become active on heating very often yield successive drops of blood which show great variation. On the other hand if too much heat is applied, the animals may become overactive and then go into a state of collapse.

In our experience the direct method described above when performed by experienced technicians is quicker, easier, and more reliable. This conforms with the published reports of Randolph (1944), Discombe (1946) and Rud (1947), who have used similar direct methods in the clinic. In general the film procedure is used to determine over-all blood changes following the injection of material having unknown physiological action. The direct method is used for the detection of quantitative variations of the eosinophils following different doses of the material.

The mice used in these experiments were C 57 Brown mice obtained from the R. B. Jackson Memorial Laboratory, Bar Harbor, Maine. Adult male mice weighing over 20 grams were found to give the most consistent results and were also the easiest to adrenalectomize. The animals were maintained on an enriched laboratory diet for one week after being received. After adrenalectomy or hypophysectomy a 1% saline solution replaced their drinking water and additional sugar was mixed in their food or placed in separate dishes.

Most of the rats were obtained from Holtzman Laboratory Animals, Inc., Madison, Wisconsin, and weighed from 100 to 200 grams.

OPERATIVE TECHNIQUES

The various operative techniques used in the mouse are very similar to those used in the rat and described by Ingle and Griffith (1942). Our technique of hypophysectomy is a modification of a procedure described by Thomas (1938). In most cases the whole operation can be performed in less than 10 minutes, with an extremely low mortality. In over 200 operations performed over a period of three years, very few of the mice ever showed an increase in weight after the operation.

The adrenalectomies can likewise be performed with little or no mortality in a one stage operation lasting about 2 minutes. The right adrenal is sometimes difficult to remove without rupturing and pieces of the capsule are often left in the body cavity. For this reason all animals must be tested first by a procedure to be described in this paper, before they can be assumed to be completely adrenalectomized.

PROCEDURE AND RESULTS

A. Eosinophil determinations in normal mice

The eosinophil count of mice as well as other animals varies

greatly from time to time depending upon the physiological state of the animal. In a series of 63 separate determinations in which undisturbed mice were removed from a cage and an eosinophil count performed within 10 minutes, we found the average count to be 409 cells per cubic millimeter. The range of variation was from 70 to 1280 cells.

However, when more than one determination was made on the same animal the number of eosinophils was found to change in a consistent manner. This is shown by the first experiment, the data of which are tabulated in Table A. Eight undisturbed mice were removed from a cage and 5 determinations of the number of circulating eosinophils were made over a period of 4 hours. The first count (called the zero hour count) was made within 10 minutes after the animals were removed from their cage. The technique for obtaining blood was followed exactly as described for the direct method. Successive determinations of the eosinophils were made after an interval of 30 minutes, 1 hour, 2 hours and 4 hours. The actual number of eosinophils per cubic millimeter is shown in the first 5 columns and the per cent change shown in the last 4 columns of Table A. It can be seen that during the first half hour there is no consistent change. In half the animals the number of eosinophils decreased slightly and in the remaining half they increased over the initial count.

TABLE A. SUCCESSIVE DETERMINATIONS OF CIRCULATING EOSINOPHILS IN NORMAL MICE

Animal	Stimulus	Eosinophil count					Per cent change*			
		0 Hr.	½ Hr.	1 Hr.	2 Hr.	4 Hr.	½ Hr.	1 Hr.	2 Hr.	4 Hr.
Normal mice	Handling and loss of blood	750	663	463	94	6	− 12%	− 38%	−87%	− 99%
		200	225	150	38	0	+ 13%	− 25%	−81%	−100%
		206	174	94	25	6	− 15%	− 54%	−88%	− 97%
		331	344	188	75	0	+ 4%	− 43%	−77%	−100%
		203	228	97	31	3	+ 12%	− 52%	−85%	− 99%
		147	353	384	60	3	+140%	+160%	−59%	− 98%
		209	228	122	41	—	+ 9%	− 42%	−80%	—
		284	419	191	44	6	+ 48%	− 33%	−85%	− 98%
Average							+ 24%	− 16%	−80%	− 99%

* Per cent change is computed as follows:

$$\left(\frac{\text{Number of cells at each successive count}}{\text{Number of cells at zero hours}} - 1\right) \times 100.$$

In the hour determination however, all but one of the mice showed a decrease in the number of circulating eosinophils. After 2 hours all the animals showed an average decrease of 80% of the initial count; after 4 hours there was a 99% decrease. Thus within a period of 4 hours the eosinophils had almost completely disappeared from the tail blood.

There are at least two possible explanations for this change in the number of eosinophils. First, a local inflammatory reaction due to successive cuts on their tails. Second, the excitement due to handling the mice might have set up an "alarm reaction" which in some way affected the eosinophil cells.

A second experiment was designed to determine whether mild

stresses, not associated with trauma and loss of blood, would cause a decrease in eosinophils. A cage containing normal mice was opened and the mice shaken out of their bedding. The animals ran around the cage in mild excitement for a few minutes and then quieted down. Four hours later a blood count was made, and the following morning a second count was taken. It can be assumed that the 18 hour count, made after the mice had been left undisturbed overnight, would be approximately the same as the count would have been, if one had been made at 0 hours. The basis for this assumption is discussed later in this paper.

It can be seen from Table B that the eosinophil count made 4 hours after mild excitement was 90% lower than a similar count made after the same mice had been left undisturbed for 14 hours. This experiment indicates that the loss of blood or local inflammation of the tail is not the major factor causing the decrease in circulating eosinophils.

TABLE B. EFFECT OF MILD STIMULATION ON THE EOSINOPHILS OF NORMAL MICE

Stimulus	Eosinophil count			%* Change
	0 Hr.	4 Hr.	18 Hr.	
Cage cleaned. Mice shaken out of cotton	—	38	374	−90%
	—	34	544	−94%
	—	228	881	−74%
	—	9	331	−97%
	—	31	303	−90%
	—	122	859	−86%
	—	6	284	−98%
Average				−90%

* Per cent change: $\left(\frac{\text{4 hour count}}{\text{18 hour count}} - 1\right) \times 100.$

A third experiment was designed to study in more detail the actual change in the eosinophil count after a single period of excitement or stress. In this experiment 7 groups of normal mice were used, 8 animals in each group. Two determinations were made of the number of circulating eosinophils in each animal. Between determinations the animals were returned to their cages for specific periods of time. The handling of the mice at the time of the first bleeding is considered a mild stress and is responsible for bringing about changes seen in the second count. See Table C. The first group had the two counts made approximately ½ hour apart. During this time there was no significant change in the number of eosinophils. In the second group, in which the eosinophil determinations were taken one hour apart, all the animals showed a significant decrease, averaging approximately 40%. In the 2 hour group there was a decrease of 49%, and in the 4 hour group the decrease averaged 83%.

TABLE C. PROGRESSIVE CHANGE IN THE NUMBERS OF CIRCULATING EOSINOPHILS IN NORMAL MICE FOLLOWING A MILD STRESS*

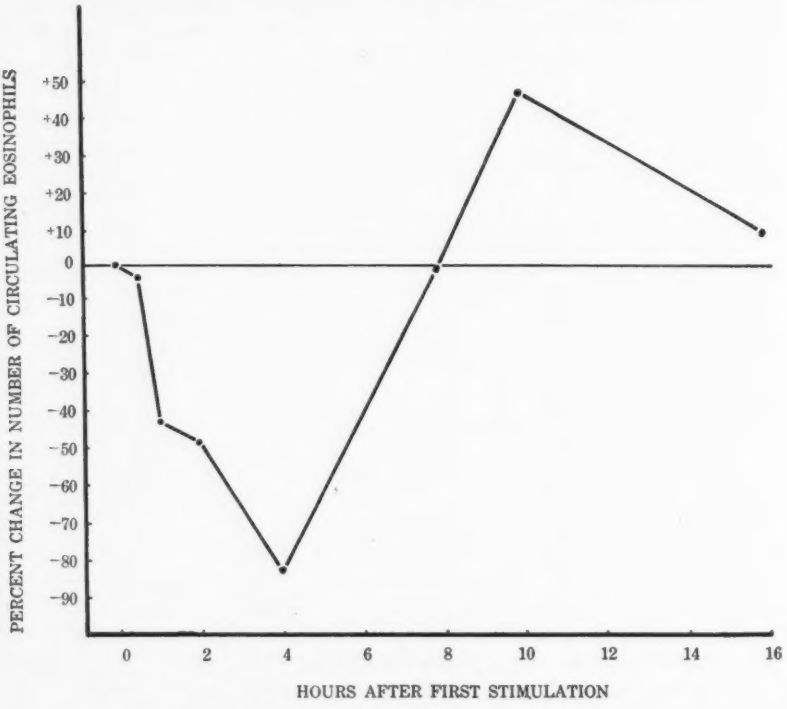
Group I			Group II			Group III			Group IV			Group V			Group VI			Group VII		
0	½ Hr.	%†	0	1 Hr.	%	0	2 Hr.	%	0	4 Hr.	%	0	8 Hr.	%	0	10 Hr.	%	0	16 Hr.	%
556	463	−17%	206	63	−69%	375	213	−43%	125	38	−70%	206	225	+9%	85	107	+ 26%	325	403	+24%
413	428	+ 4%	881	438	−50%	303	125	−59%	219	36	−84%	88	153	+73%	275	394	+ 43%	284	194	−32%
806	750	− 7%	1281	984	−23%	578	266	−54%	144	13	−91%	94	163	+74%	163	416	+155%	88	116	+32%
141	122	−13%	622	528	−15%	278	141	−49%	356	13	−96%	188	216	+15%	225	259	+ 15%	413	141	−66%
409	444	+ 9%	750	463	−38%	331	153	−54%	213	88	−59%	403	184	−54%	191	381	+100%	150	213	+42%
750	663	−11%	200	150	−25%	381	225	−41%	575	31	−95%	406	82	−80%	141	156	+ 11%	166	188	+13%
200	225	+13%	206	94	−54%	544	278	−49%	625	131	−79%	116	76	−34%	338	384	+ 14%	76	128	+68%
206	175	−15%	331	188	−43%	1081	647	−40%	400	38	−90%	141	128	− 9%	703	769	+ 9%	107	94	−12%
Average		− 5%	Average		−40%	Average		−49%	Average		−83%	Average		− 1%	Average		+ 47%	Average		+ 9%

* These mice were removed from their cages, warmed, and a sample of blood taken from their tails. No other stimulus was applied. The mice were then put back in their cages for specific periods of time, after which a second sample was taken.

† Per cent change: $\left(\frac{\text{Number of cells at each successive count}}{\text{Number of cells at zero hours}} - 1\right) \times 100$

In the 5th group, in which the second eosinophil determination was made 8 hours after the first, half of the animals showed an increase over the original zero hour count. In the 10 hour group all the animals showed an increase averaging 47%. Finally, in the last group there was a large variation, but the average of the 8 animals at 16 hours was approximately the same as the determination made at zero hours.

The changes shown in Table C are plotted in Figure 1.



CHANGE IN NUMBER OF CIRCULATING EOSINOPHIL CELLS IN NORMAL MICE FOLLOWING MILD STRESS.

FIG. 1

In summary, it can be emphasized that a mild stress produces a great decrease in the number of circulating eosinophils which lasts over a period of approximately 8 hours. Following this there is an increase in the number of circulating eosinophils and a return to normal by approximately 16 hours after the initial stimulation.

It would be expected, that if more than one mild stimulation were given, the effect would be cumulative. To test this hypothesis mice which had had two determinations made within ½ hour were

checked again at 8 hours. In these animals the number of eosinophils still showed a decrease of 96%, whereas the average from a similar group of animals which had had only one stimulation returned to approximately the original level by 8 hours. By 20 hours the number of eosinophils was approximately normal again in the group with the double stimulation.

TABLE D. EFFECT OF DOUBLE STIMULATION ON THE EOSINOPHILS OF NORMAL MICE

Stimulus	Eosinophil count				Per cent change*		
	0 Hr.	½ Hr.	8 Hr.	20 Hr.	½ Hr.	8 Hr.	20 Hr.
Handling and loss of blood	556	463	3	278	−17%	− 99%	−50%
	1275	1300	16	1077	+ 2%	− 99%	−15%
	1047	1263	19	1472	+21%	− 98%	+41%
	413	428	59	381	+ 3%	− 86%	− 8%
	806	750	3	681	− 7%	− 99%	−16%
	141	122	0	206	−13%	−100%	+46%
	409	444	19	578	+ 9%	− 95%	+41%
Average					0%	− 96%	+ 6%

* Per cent change:

$$\left(\frac{\text{Number of cells at each successive count}}{\text{Number of cells at zero hours.}} - 1\right) \times 100.$$

TABLE E. EFFECT OF EPINEPHRINE INJECTIONS ON THE EOSINOPHILS OF NORMAL ANIMALS

Animal	Stimulus	Eosinophil count		Per cent change*
		0 Hr.	4 Hr.	
Intact mice	5 gamma Epinephrine	763	38	− 95%
		238	6	− 97%
		750	6	− 99%
		200	0	−100%
		206	6	− 97%
		331	0	−100%
		1060	19	− 98%
Average				− 98%
Intact rats	200 gamma Epinephrine	369	34	− 91%
		209	103	− 51%
		188	16	− 91%
		363	59	− 84%
Average				− 79%

* Per cent change: $\left(\frac{\text{4 hour count}}{\text{0 hour count}} - 1\right) \times 100.$

If this decrease in eosinophils is part of the mechanism by which animals respond to stress, then injections of epinephrine should produce approximately the same effect. To determine whether this was true, 8 mice were injected with 5 gamma of epinephrine, and eosino-

phil determinations made before the injection and 4 hours later. The results obtained are shown in Table E. All 8 animals showed a decrease averaging 98%. Epinephrine injected into rats also produced approximately the same effect. In 4 animals the average decrease was 79% at 4 hours.

In 5 separate experiments described above, the numbers of eosinophils in mice and rats were found to vary in a consistent manner following stress. There was a decrease in the number of circulating eosinophils beginning within one hour after the stress was first applied, and lasting for 7 hours or more depending upon the degree and amount of stress. If the stress is sufficiently great there will be an almost complete disappearance of the eosinophils from the tail blood. Following this period of eosinopenia there was a second period in which an eosinophilia occurred. In most animals this disappeared by 16 hours.

These data indicate that a great deal of consideration should be given to the procedures used to obtain blood from normal mice and rats, if an accurate determination is to be made. The animals should not be handled or disturbed for at least 16 hours prior to a blood sampling. Once the mice are disturbed by removing them from their cages, etc., the blood sample should be taken as soon as possible.

The authors would like to point out that the eosinophil picture following mild stress is characteristic for the C 57 Brown mice used, and might be quite different in other strains of mice.

B. Eosinophil determinations in adrenalectomized mice

In a series of 454 determinations of the level of circulating eosinophils in adrenalectomized mice, the average number of cells was found to be 349 per cubic millimeter. There was a wide variation of from 10 to 1653 eosinophils. Thus the average count and the range of variability in the adrenalectomized mouse was not significantly different from that of the intact animal.

One of the most important factors to consider in these animals is

TABLE F. EFFECT OF MILD STIMULATION ON THE EOSINOPHILS OF ADRENALECTOMIZED MICE

Animal	Stimulus	Eosinophil count				Per cent change*		
		0 Hr.	2 Hr.	4 Hr.	8 Hr.	2 Hr.	4 Hr.	8 Hr.
Adrenalectomized mice	Handling and loss of blood	134	131	138	144	− 2%	+ 3%	+ 7%
		331	297	347	306	−10%	+ 5%	− 8%
		76	138	225	228	+82%	+196%	+200%
		219	213	353	441	− 3%	+ 61%	+101%
		406	469	600	638	+16%	+ 48%	+ 57%
		234	266	316	209	+14%	+ 35%	− 11%
Average						+16%	+ 58%	+ 59%

* Per cent Change:

$$\left(\frac{\text{Number of cells at each successive count}}{\text{Number of cells at zero hours}} - 1\right) \times 100.$$

the change in the number of circulating eosinophils after various stress stimuli. In the first experiment 6 mice were adrenalectomized and maintained by the addition of 1% salt to their drinking water. Three days later, 4 successive blood determinations were made over a period of 8 hours. The data obtained are shown in Table F. It will be noted that the number of eosinophils in the circulation of the mouse's tail actually increased in the succeeding determinations. In all these animals the removal of the adrenal prevented the eosinopenia which occurs in normal animals following stress.

TABLE G. EFFECT OF EPINEPHRINE INJECTIONS ON THE EOSINOPHILS OF ADRENALECTOMIZED MICE

Animal	Stimulus	Eosinophil count		Per cent change*
		0 Hr.	4 Hr.	
Completely adrenalectomized mice	5 gamma Epinephrine	181	263	+45%
		144	250	+74%
		244	263	+ 8%
		313	488	+56%
		363	438	+21%
		494	519	+ 5%
		188	300	+60%
		294	513	+74%
		Average		+43%

* Per cent change: $\left(\frac{\text{4 hour count}}{\text{0 hour count}} - 1\right) \times 100$.

If 5 gamma of epinephrine is injected into each of the adrenalectomized mice, the number of eosinophils also increases over a 4 hour period. These data are shown in Table G. The same general increase (52%) also occurred in 4 adrenalectomized rats following 200 gamma of epinephrine, subcutaneously. Thus stress and epinephrine injections do not cause a decrease in the number of circulating eosinophils in adrenalectomized mice and rats.

In the next experiment the effects of an extract of the adrenal cortex was tested in adrenalectomized mice. Five mice received an injection of 0.05 cc. Lipo-Adrenal Cortex (Upjohn Company) and eosinophil determinations made over a period of 12 hours. The film method described earlier was used to determine the number of eosinophils.

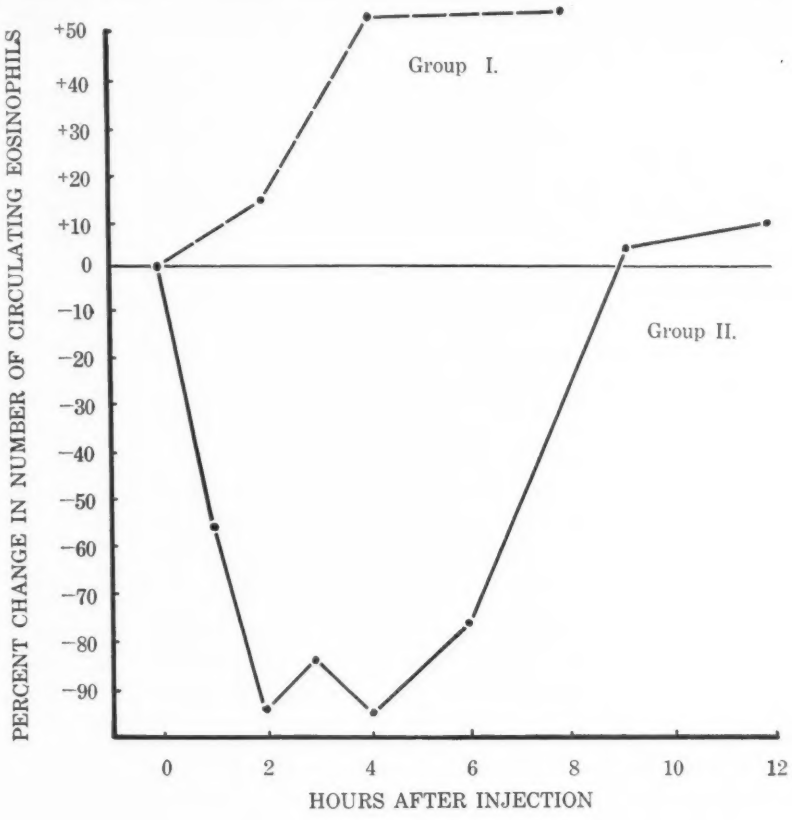
The data shown in Table H and Figure 2 show that the cortin produces a marked decrease in the eosinophil count beginning within the first hour, and lasting for more than 6 hours. By 9 and 12 hours the number of circulating eosinophils has returned to normal. Thus in completely adrenalectomized animals eosinopenia can be obtained by injecting cortical extracts. The time of response and type of curve obtained is almost identical to that produced in the normal animal after stress.

However, if mice are subjected to a mock operation, unilateral

TABLE H. EFFECT OF ONE INJECTION OF CORTIN ON THE EOSINOPHILS OF ADRENALECTOMIZED MICE

Injection	Eosinophil count								Per cent change*						
	0	1 Hr.	2 Hr.	3 Hr.	4 Hr.	6 Hr.	9 Hr.	12 Hr.	1 Hr.	2 Hr.	3 Hr.	4 Hr.	6 Hr.	9 Hr.	12 Hr.
0.05 cc. Cortin	400		36		26	0	807	453		− 91%		− 93%	−100%	+102%	+13%
	638		61		53	226	126	518		− 90%		− 92%	− 58%	− 80%	−19%
	647		0		0	82	306	291		−100%		−100%	− 87%	− 53%	−55%
	445	173		51		134	514	632	−61%		−89%		− 70%	+ 16%	+42%
	443	225		113		15	593	750	−49%		−74%		− 97%	+ 34%	+69%
Average									−55%	− 94%	−82%	− 95%	− 82%	+ 4%	+10%

* Per cent change: $\left(\frac{\text{Number of cells at each successive count}}{\text{Number of cells at zero hours}} - 1\right) \times 100.$



CHANGE IN NUMBER OF CIRCULATING EOSINOPHIL CELLS IN ADRENALECTOMIZED MICE.

Group I. Following mild stress.
Group II. Following a subcutaneous injection of 0.05 cc Cortin (Lipo-Adrenal Cortex)

FIG. 2

adrenalectomy, or if remnants of one or both adrenals are left within the body cavity at the normal site or are transplanted to the spleen or kidney, then a decrease in eosinophils will follow the epinephrine injections. This is shown in Table I. It will be noted that in many of these animals in which there has been a regeneration of the adrenals, a typical eosinopenia does not occur. There is an average decrease of 65%, and many animals show only a 30 or 40% decrease, as opposed

TABLE I. EFFECT OF EPINEPHRINE INJECTIONS ON THE EOSINOPHILS OF MICE WITH PARTIAL REGENERATION OF ONE OR BOTH ADRENALS

Animal	Stimulus	Eosinophil count		Per cent change*
		0 Hr.	4 Hr.	
Adrenalectomized mice in which there has been partial regeneration of one or both adrenals	5 gamma Epinephrine	769	75	−90%
		119	31	−74%
		138	94	−32%
		407	250	−39%
		150	9	−94%
		241	110	−54%
		275	169	−39%
		450	113	−75%
		Average		−62%

* Per cent change: $\left(\frac{\text{4 hour count}}{\text{0 hour count}} - 1\right) \times 100$.

to a 98% decrease in normal animals (Table E). The tentative conclusion drawn from this is that the regenerated adrenal is not as efficient as normal adrenals. Furthermore, the data indicate that the pattern of eosinophil change following epinephrine can be used to determine whether an animal is completely adrenalectomized.

To test this last suggestion an experiment was set up to determine whether there was any correlation between the response of the eosinophils to epinephrine and the ability of the mice to live after removal of salt from their drinking water. Thirty-six animals were adrenalectomized and maintained for at least 30 days on a 1% saline solution. Five gamma of epinephrine was injected and an eosinophil count taken before injection and 4 hours later. On the basis of their response the mice could be arranged into 3 groups: Group I. Either no change or an actual increase in number of eosinophils during the 4 hours after epinephrine. Group II. Slight decrease of from −1 to −30%. Group III. A greater decrease of from −31 to −100%.

TABLE J. CORRELATION OF THE EOSINOPHIL RESPONSE OF ADRENALECTOMIZED MICE TO EPINEPHRINE AND THE ABILITY OF THESE MICE TO LIVE AFTER REMOVAL OF SALT FROM THEIR DRINKING WATER

Group	Per cent change* in eosiniphils after epinephrine	Total No. animals	Number dead after removing salt			Total dead	Total alive
			0–10 day	10–20 day	20–30 day		
I	0 to + 92%	15	12	2	0	14	1
II	− 1% to − 30%	4	2	0	1	3	1
III	−31% to −100%	17	0	0	1†	1	16

* Per cent change is computed as follows: $\left(\frac{\text{4 hour count}}{\text{0 hour count}} - 1\right) \times 100$.

† This animal at autopsy had focal areas of purulent material throughout the liver. Death probably caused by infection.

The mice were then placed in a large cage and fed a normal diet without additional saline. Daily checks were made of the cage and all deaths noted. The results are tabulated in Table J.

In Group I, 14 of the 15 mice died within 20 days. The remaining mouse was reinjected with epinephrine and this time a decrease occurred indicating that the adrenal was functioning. In Group II, 3 of the 4 animals died within 30 days. In Group III, only one animal died, apparently from an infection rather than adrenal insufficiency.

In summary, it is concluded that animals showing marked decrease in eosinophils after epinephrine injections had sufficient regeneration of the adrenal cortex to maintain them without additional saline in their drinking water. Mice which showed only a slight decrease, or an increase in eosinophils after epinephrine did not have sufficient regeneration of the cortex to maintain themselves when the saline was removed.

The authors have supplemented this experiment by autopsying many other mice. Animals which responded to epinephrine were immediately autopsied and in many cases small remnants of the adrenal tissue were seen. In a few cases, however, no actual adrenal tissue could be found macroscopically. In these animals it was assumed that adrenal rests probably had occurred in some of the organs, such as the gonads. In no case has adrenal tissue been found in animals which did not respond to epinephrine.

C. Eosinophil determinations in splenectomized mice

In a series of 13 separate determinations the number of circulating eosinophils in undisturbed splenectomized mice was found to average approximately 577 cells. The range of variability was from 169 to 1653 cells. This is not considered a significant increase over the count obtained for normal animals. However, the mice had only been splenectomized for a period of two weeks.

In the first experiment successive blood counts were made over a period of 4 hours on 5 splenectomized mice. The data obtained are shown in Table K. In these animals a rapid decrease in the number of circulating eosinophils occurred which was similar to the response of intact animals shown in Table A. This indicates the spleen is not involved in the rapid decrease in circulating eosinophils following mild stress.

In a second experiment 6 mice were adrenalectomized and splenectomized in a one stage operation. In these animals the average number of eosinophils increased after mild stress as shown in Table K. This response was similar to that obtained from adrenalectomized mice (Table F). These data suggest that the spleen is not involved in the increase in numbers of eosinophils which occurs in adrenalectomized animals.

TABLE K. EFFECT OF MILD STRESS ON THE EOSINOPHILS OF SPLENECTOMIZED AND ADRENALECTOMIZED-SPLENECTOMIZED MICE

Animal	Stimulus	Eosinophil count					Per cent change*			
		0 Hr.	1 Hr.	2 Hr.	4 Hr.	8 Hr.	1 Hr.	2 Hr.	4 Hr.	8Hr.
Splenectomized mice	Handling and blood loss	709	472	163	128		−33%	−70%	−82%	
		1653	472	88	91		−71%	−95%	−94%	
		906	506	85	16		−44%	−91%	−98%	
		169	150	69	3		−11%	−59%	−98%	
		500	544	200	6		+ 9%	−60%	−99%	
Average							−30%	−76%	−94%	
Adrenalectomized-splenectomized mice.	Handling and blood loss	331		428	481	547		+29%	+45%	+ 65%
		563		563	969	753		0	+72%	+ 34%
		1038		1203	1131	1106		+16%	+ 9%	+ 7%
		178		341	347	406		+92%	+95%	+128%
		979		1266	1141	1406		+29%	+17%	+ 44%
		288		269	281	356		− 7%	− 2%	+ 24%
Average								+27%	+39%	+ 50%

* Per cent change: $\left(\frac{\text{Number of cells at each successive count}}{\text{Number of cells at zero hours}} - 1\right) \times 100.$

D. Eosinophil determination in hypophysectomized mice

In a series of 41 different determinations made in undisturbed hypophysectomized mice, we found the average eosinophil count to be 290 cells per cubic millimeter. The range of variation was from 28 to 866 cells. This count is not considered significantly different from that of normal mice.

The effects of handling, epinephrine, and ACTH injections are shown in Table L. It can be seen readily that handling and epinephrine injections caused a decrease in the number of circulating eosinophil cells during a 4 hour period. This decrease occurred in all animals during a period of from 3 to 14 days after hypophysectomy. Injections of 50 gamma of ACTH produced a much greater decrease, averaging 87%, during the same period of time.

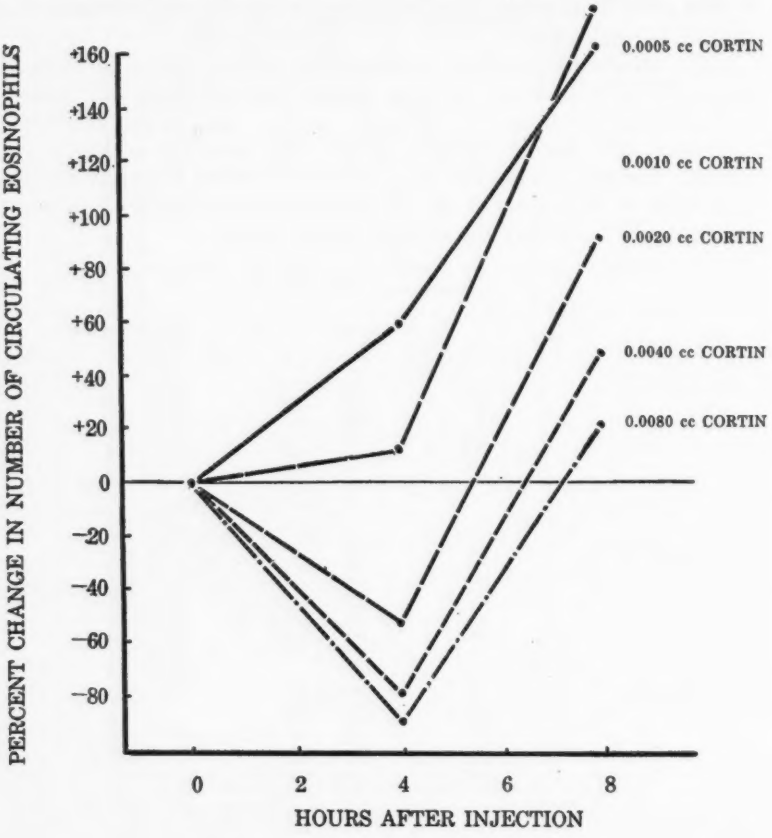
TABLE L. EFFECTS OF VARIOUS STIMULI ON THE EOSINOPHILS OF HYPOPHYSECTOMIZED MICE

Stimulus	Days A/P	Eosinophil count				Per cent change		
		0 Hr.	2 Hr.	4 Hr.	8 Hr.	2 Hr.	4 Hr.	8 Hr.
Handling and blood loss	3	275		222	381		− 19%	+ 39%
	3	491		453	488		− 8%	− 1%
	3	613		488	541		− 20%	− 12%
	3	603		344	353		− 43%	− 41%
	3	250		147	181		− 41%	− 28%
	7	147		82	141		− 44%	− 4%
	7	166		44	297		− 73%	+ 79%
	7	303		188	328		− 38%	+ 8%
	7	28		3	31		− 89%	+ 11%
	Average						− 42%	+ 6%
5 gamma Epinephrine	7	107		56			− 48%	
	7	103		56	241		− 46%	+134%
	7	241		94	275		− 61%	+ 14%
	7	266		110	328		− 62%	+ 23%
	7	172		110	110		− 36%	− 36%
	7	209		144	247		− 31%	+ 18%
	7	144		110	138		− 24%	− 4%
	Average						− 44%	+ 25%
5 gamma Epinephrine	14	113		38	69		− 66%	− 39%
	14	328		250	291		− 24%	− 11%
	14	403		313	391		− 22%	− 3%
	14	197		9	85		− 95%	− 57%
	14	313		141	—		− 55%	—
	14	534		294	394		− 45%	− 26%
	14	191		44	216		− 72%	+ 13%
	Average						− 54%	− 21%
50 gamma ACTH	4	147	41	53	191	−72%	− 74%	+ 30%
	4	219	50	31		−77%	− 86%	
	4	134	28	19		−79%	− 86%	
	4	172	16	0		−91%	−100%	
	4	82	3	13		−96%	− 84%	
	4	266	85	13		−69%	− 95%	
	4	234	113	44		−52%	− 81%	
	Average					−77%	− 87%	+ 30%

E. Effect of various steroid materials on the eosinophil count of adrenalectomized mice and rats

It has already been shown in section B, Table H, that extracts of the adrenal cortex will produce temporary eosinopenia in adrenalectomized mice. It is of importance to determine whether this eosinopenia is a specific quantitative response.

The first experiment was designed to determine whether varying doses of cortin would produce different levels of response in adrenalectomized mice. The cortin was diluted with corn oil so that each animal received 0.03 cc. injection of oil subcutaneously in the dorsal region



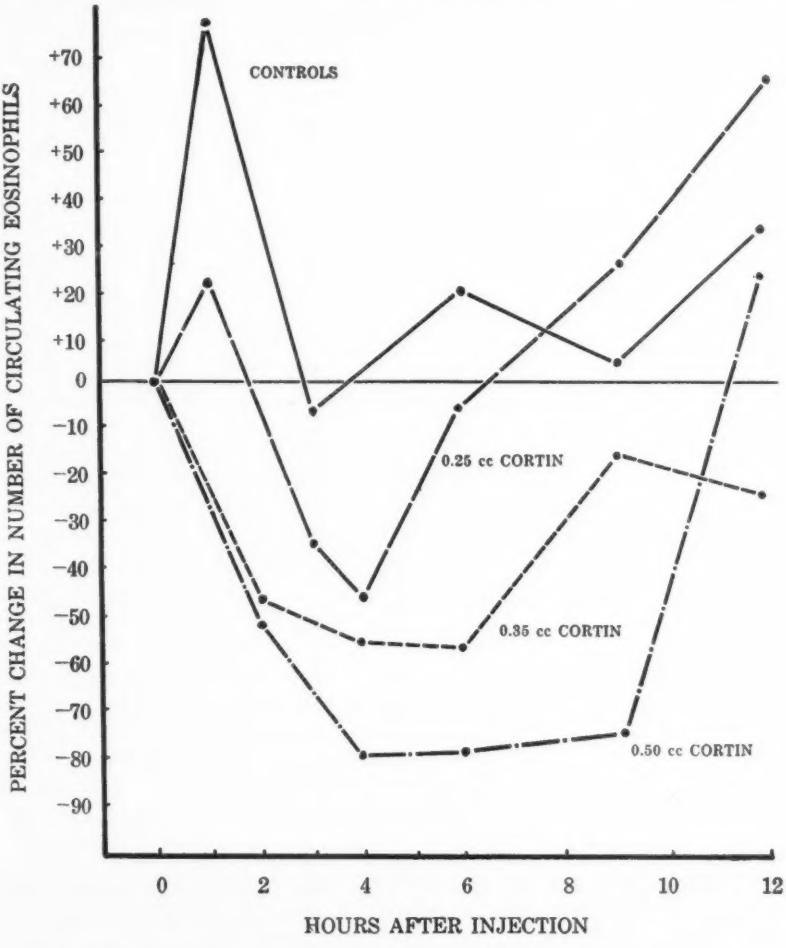
CHANGE IN NUMBER OF CIRCULATING EOSINOPHIL CELLS FOLLOWING A SUBCUTANEOUS INJECTION OF VARYING DOSES OF CORTIN IN ADRENALECTOMIZED MICE.

FIG. 3

of the back. Eosinophil determinations were made before the injections and at 4 and 8 hours afterwards. The data from 29 mice were calculated and plotted in Figure 3. The smallest dose, equal to 0.0005 cc. of the original extract, did not produce an eosinopenia, but caused instead an average increase of 59% and 162% at 4 and 8 hours respectively. The next dose produced a decrease in 3 out of the 6 ani-

mals and the average was 8% increase at 4 hours. The third and all larger doses produced a marked decrease in all of the animals at the 4 hour determination. At the 8 hour determination the averages of all groups were higher than the initial zero hour count. It can be clearly seen in Figure 3 that the extent of the decrease in eosinophils was correlated with the amount of cortin injected.

The same type of response occurred in rats, except that the amount of cortin needed to produce the change was approximately



CHANGE IN NUMBER OF CIRCULATING EOSINOPHIL CELLS FOLLOWING A SUBCUTANEOUS INJECTION OF VARYING DOSES OF CORTIN IN ADRENALECTOMIZED RATS.

FIG. 4

100 times greater than that used in the mouse. See Figure 4. It can be noted that as the dose of cortin is increased, the per cent decrease in the circulating eosinophils becomes much greater, and the period of eosinopenia also becomes longer. These data are based upon averages obtained from 24 rats.

It is apparent from the data just described that mice are much

TABLE M. EFFECT OF VARIOUS STEROID MATERIALS ON THE EOSINOPHILS OF ADRENALECTOMIZED MICE

Material injected	Dose	Eosinophil count			Per cent change*	
		0 hr.	4 hr.	8 hr.	4 hr.	8 hr.
Theelin	33γ	125	119	238	− 5%	+ 90%
	33γ	344	238	331	− 31%	− 4%
	33γ	350	681	606	+ 95%	+ 73%
	33γ	206	369	389	+ 78%	+ 89%
	Average				+ 35%	+ 62%
Testosterone Propionate	750γ	238	555	252	+133%	+ 6%
	750γ	467	421	614	− 10%	+ 31%
	750γ	197	107	260	− 46%	+ 32%
	Average				+ 26%	+ 23%
Desoxycorticosterone	150γ	244	88	106	− 64%	− 57%
	150γ	413	94	319	− 72%	− 23%
	150γ	206	75	256	− 64%	+ 24%
	Average				− 67%	− 19%
	25γ	481	581	363	+ 21%	− 25%
	25γ	250	213	281	− 15%	+ 12%
	Average				+ 3%	− 7%
Compound E	3γ	250	6	206	− 98%	− 18%
	3γ	150	0	162	−100%	+ 8%
	3γ	125	12	218	− 90%	+ 74%
	3γ	350	19	681	− 95%	+ 95%
	Average				− 96%	+ 40%
Compound A	25γ	116	31	237	− 74%	+103%
	25γ	338	31	356	− 91%	+ 5%
	25γ	263	56	406	− 79%	+ 54%
	Average				− 81%	+ 54%

* Per cent change: $\left(\frac{\text{4 or 8 hour count}}{\text{0 hour count}} - 1\right) \times 100.$

more sensitive to cortin than rats. In both animals the amount of decrease in the circulating eosinophils is correlated with the dose of cortin given. A brief period of eosinophilia usually follows the eosinopenia.

Various other steroids and oils have been injected to determine their effect on the circulating eosinophils. The data obtained after

using a few of these materials are shown in Table M. The eosinophil counts were made by the film procedure described earlier. Theelin (Ketohydroxy estratriene) and testosterone propionate have no effect on the circulating eosinophil numbers. A relatively large dose of desoxycorticosterone produced an average decrease of 67%, whereas smaller doses had no effect. The eosinophils seem to be extremely sensitive to oxycorticosteroids such as Compound E (11 dehydro-17-hydroxy-corticosterone). As little as 3 gamma of the Compound E dissolved in acetone and corn oil produced a maximum eosinopenia (96% decrease) in 4 hours. In other experiments to be published later, less then one gamma was found to be effective in reducing the eosinophil count.

Compound A (Dehydro-corticosterone) decreased the numbers of eosinophils, but was not nearly so effective as Compound E.

DISCUSSION

This paper has presented two techniques for the determination of circulating eosinophils in the mouse and rat. The direct procedure is based upon the principle that the eosinophil is the most resistant cell of the blood. A 15% acetone solution used in a 1:20 dilution with blood gave the highest and most consistent eosinophil count. In this mixture of blood and diluent, all the red blood cells and the majority of white blood cells except the eosinophil cells were either made indistinct or destroyed. The second procedure used was similar to techniques used in routine clinical and animal hematology. The only modification was in an improved staining procedure. Wright's and Giemsa's Blood Stains were used together in a coplin jar to produce a better staining of the eosinophil granules in the mouse. Total leukocyte counts were determined in a 4% acetic acid solution, and differential counts were made from stained blood films.

In general the film procedure is used to determine over-all blood changes following the injection of material having unknown physiological action. The direct method is quicker, easier, and more reliable and is used for the detection of quantitative variations of the eosinophils following different doses of the adrenal cortical hormone, etc.

An estimate of the over-all error of the direct technique was obtained by making counts of successive drops of blood taken directly from a mouse's tail. The standard error was found to be 1.7 times the square root of the number of cells counted. This includes the variation in eosinophils in successive drops of blood obtained from the tail as well as errors in technique, instruments, etc.

The results obtained in this paper clearly indicate that there is no significant difference in the initial eosinophil counts of normal, adrenalectomized, or hypophysectomized mice, just after removal from their cages. However, succeeding blood determinations following mild stress or epinephrine injections showed a marked difference

between the three groups of animals. In normal intact mice there is a marked eosinopenia lasting for approximately 7 hours, followed by a slight eosinophilia. See Figure 1. In the adrenalectomized mice a slight eosinophilia occurs. Eosinopenia does not occur except after injections of adrenal cortical extracts. See Figure 2. In the hypophysectomized mice a decrease in the number of eosinophils occurred, but it was not so marked as in the normal animals. However, a complete eosinopenia was obtained by injections of ACTH.

These data can best be interpreted as follows: Stress or epinephrine injections produce a release of oxycorticosteroids from the adrenal cortex which causes a decrease in the number of circulating eosinophils. The spleen is not involved in these reactions.

In a recent review, Long (1947) pointed out that there are three possible paths through which the epinephrine may act to cause a release of cortin:

1. Directly on the pituitary gland causing a release of ACTH which acts upon the adrenal cortex.
2. By producing a drop in the level of cortical hormone in the blood, which in turn produces an increased release of ACTH by the pituitary.
3. Directly on the adrenal cortex causing a release of the cortical hormone.

The data presented in this paper indicate that there may be a combination of two or more of these paths in the mouse. As already indicated, epinephrine produces a marked decrease in circulating eosinophils if both the pituitary and adrenal are present. If the adrenal cortex is removed, epinephrine injections will not produce the decrease. If the pituitary is removed, epinephrine produces a decrease in the eosinophils, but a complete eosinopenia does not occur. This indicates that in hypophysectomized animals the epinephrine acts directly on the adrenal cortex to produce a release of cortical hormone. The amount of hormone released is small in comparison with the amount released after ACTH is injected.

These observations agree with published results of Vogt (1944). She was able to obtain increased cortical hormone output in a dog following perfusion of the adrenal with epinephrine. The effect was not mediated through the pituitary. However, Long and Fry (1945) noted that there was no measurable decrease in cholesterol or ascorbic acid in the adrenals of hypophysectomized rats following epinephrine injections. It is possible that the amount of hormone released is too small to be detected by their methods.

There is a second possible interpretation of these results. Experiments of Dean and Greep (1948) have suggested that release of desoxycorticosteroids is not under pituitary control in the rat. If the epinephrine produced a release of large amounts of this material, then it is conceivable that a decrease of the eosinophils may follow.

The use of epinephrine injections to test the completeness of adrenalectomy should prove to be very useful. It can be used to screen a group of animals to determine which ones have tissue capable of producing cortical hormones. This is particularly important in various assay procedures. It is now possible to eliminate all incompletely adrenalectomized animals before an experiment is set up on the basis of their eosinophil response after epinephrine. This is much quicker and easier than the transplantation procedure used by Kroc (1942). Furthermore, the relation of the hormone secretion and regeneration can now be followed. For example, it has been observed that in a group of animals very few will show a positive test for functional adrenal tissue during the first few days after adrenalectomy. However, after that, a slight eosinopenia begins to occur in some after epinephrine. This response becomes progressively greater during the following weeks, indicating that an animal should be tested for adrenal cortical tissue each time it is to be used in an assay procedure.

In many of the animals which have regenerated adrenal tissue a complete eosinopenia is not obtained even after 3 months or more after adrenalectomy and regeneration. Following a 5 gamma injection of epinephrine the eosinophils do not decrease in the same proportion as in normal animals. Yet these animals survive after removal of salt from their drinking water, and under good conditions will grow and appear healthy. However, following a mild stress, these animals seem to die very easily. For example, after a series of blood determinations were made, many of the mice went into shock and died.

In a similar manner, animals which had adrenal cortical transplants in the spleen and kidney do not respond to adrenalin by complete eosinopenia (unpublished data). In 1940 Eversole, Edelmann and Gaunt pointed out that rats, with adrenals transplanted to various parts of the body, could survive removal of salt from their drinking water, but could not cope with intraperitoneal injections of water in the same manner as normal animals. This is in agreement with results obtained by many other authors, indicating that a regenerated cortex is functionally inadequate when the animal is subjected to intense or prolonged stress (Wyman and Tum Suden, 1937; Williams, 1947; Turner, 1948).

At the present time the authors are unable to explain the increase in the level of eosinophils in adrenalectomized animals. It has been noted in many mice and rats that an increase of as much as 200% is common following an initial eosinophil determination. This increase may be a local effect caused by cutting the tail of the animals, or possibly by a general hemoconcentration, or by stimulation of the bone marrow by epinephrine or some other compound. However, the spleen is not responsible for this increase, inasmuch as it occurs in splenectomized-adrenalectomized mice.

Graduated doses of an adrenal cortical extract produce different degrees of eosinopenia in adrenalectomized rats and mice. The eosinopenia is transitory, usually lasting less than 8 hours, and is followed by an eosinophilia. In all the materials used, this reaction was found to be specific for the adrenal cortical hormones, especially the oxycorticosteroids. The response was not obtained when sex hormones, oils, and various other materials were injected.

The factors presented in this paper form a background for a suggested technique for measuring exogenous adrenalcortical extracts, to be published in the near future.

SUMMARY

A direct procedure for determining eosinophils in mice and rats is presented along with a modified procedure for staining blood films in coplin jars.

A method of obtaining blood from mice is described in detail. This method was found to give reasonably consistent eosinophil counts when succeeding drops of blood were taken from the tail. The standard error of this procedure was found to be 1.7 times the square root of the number of eosinophils counted.

These techniques were applied to mice and rats and the following results obtained:

In normal mice, mild stress or epinephrine injections produced a great decrease in the number of circulating eosinophils. This eosinopenia lasted for approximately 7 hours and was followed by a period of eosinophilia. By increasing the amount of stress, a greater decrease in eosinophils occurred lasting over a longer period of time.

Removal of the adrenal cortex prevented the eosinopenia due to stress and epinephrine; instead a slight eosinophilia occurred after each determination. However, the eosinopenia was obtained after injections of adrenal cortical hormones. The degree of response was correlated with the amount of hormone injected. Sex hormones, oils, and low doses of desoxycorticosteroids did not produce an eosinopenia in adrenalectomized mice.

Evidence was presented which indicates that the change in the number of circulating eosinophils can be used to determine whether the animal has functional cortical tissue present. If a decrease of 30% or more in the number of circulating eosinophils occurs, then there is sufficient adrenal cortical tissue present to maintain the mouse after removal of the excess salt in its drinking water.

The spleen is not involved in the rapid decrease in the circulating eosinophils, nor in the eosinophilia of adrenalectomized mice.

In hypophysectomized mice, stress and epinephrine produced a decrease of from 40% to 50% in the number of circulating eosinophils. ACTH produced a greater effect, averaging an 87% decrease when 50 gamma was injected.

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EXCRETION OF GLUCOCORTICOIDS IN THE NEWBORN[1]

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PHILADELPHIA

THE ADRENAL gland of the newborn infant differs greatly from that of the adult person. At birth the adrenals are relatively large structures and the cortex is composed of an outer or true cortical part and an inner part, the fetal cortex. This fetal cortex grows rapidly during the last trimester of fetal life and at birth or just previous to birth begins to degenerate. According to Benner (1940) this process is slow for the first 3 days of life but after that time proceeds rapidly for 2 to 3 weeks. This phenomenon was first observed by Scheel in 1908 and although numerous investigations have been carried out since that time, the function of the fetal cortex and the significance of its rapid involution are still not understood.

Grollman (1936) and Broster (1937) have suggested that this zone might be androgenic tissue, but attempts by Gersh and Grollman (1939) and by Carnes (1940) to demonstrate the presence of androgenic substance in the fetal adrenal have failed. In the following study an attempt has been made to determine whether the fetal cortex produces glucocorticoids, those adrenal hormones which promote the conversion of body protein to carbohydrate. As glucocorticoids are excreted in urine their measurement affords a relative means of evaluating the activity of the adrenal cortex with regard to this function.

CLINICAL MATERIAL AND METHODS

A total of twelve healthy full term and seven premature babies were studied at the Hospital of the University of Pennsylvania, Philadelphia. As only very small amounts of active adrenal sustances are excreted by the newborn, in each case the urine had to be collected over a period of 3 to 4 days in order to obtain a satisfactory bioassay. In thirteen of these infants the urine was completed by the sixth day of life. In the others the collection was made at varying times between the ninth and thirty-seventh day of life. The prematurity of the babies varied from four to eight weeks and all infants

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weighed less than 2500 gms. and were considered to be well at the time of collection of the urine.

Two premature babies born of diabetic mothers and suffering from atelectasis were studied at the Royal Victoria Montreal Maternity Hospital. These cases are included through the courtesy of Dr. C. H. Read.

Baby M, a male, was delivered by Caesarian section about one month prior to term. At about 13 hours of age, he began to have moderately labored respirations. There was relative dullness over the left lung field with almost absent breath sounds. A clinical diagnosis of atelectasis was confirmed by radiological examination. The infant was put in an incubator and given continuous oxygen. The following day there was considerable improvement and on the fifth day of life the lungs were fully expanded. Urine collection began 4 hours after birth and was continued for seventy-two hours.

Baby B, a male was delivered by Caesarian section one month prior to term. The mother had been a severe diabetic for 10 years. At birth the baby was limp with a moderate amount of edema. He was placed in an incubator and given oxygen. About one half hour later, the baby became dyspneic and cyanosis was apparent if the oxygen was stopped. The clinical findings of complete collapse of the left lung with partial collapse of the right lung were confirmed by x-ray. There was slight improvement on the following day with some aeration of the right lung, and gradual improvement from then on, but the lungs were not clear clinically and radiologically until the seventh day of life. The first urine collection began at 6 hours after birth and was continued for seventy-two hours. Subsequent collections were made at the seventh, eleventh and fifty-fourth days of life.

On the possibility that the placenta might be storing glucocorticoids and allowing them to pass to the fetus, three placentae were examined separately for their content of glucocorticoids. The whole placenta was mixed in a Waring blender with $1\frac{1}{2}$ times its volume of water. The mixture was acidified with hydrochloric acid to pH_2 and from that point treated in a similar manner as the urine specimens. The weight of the placentae varied from 240 gm. to 500 gm.

The glucocorticoids were determined by the bioassay method of Venning, Kazmin and Bell (1946) which is dependent upon the gylcogen deposited in the livers of fasted adrenalectomized mice. The results are expressed in terms of the standard 11-dehydro-17 hydroxy corticosterone or cortisone. Previously the term glycogenic units was used.

RESULTS

The results on the healthy babies are charted in Fig. 1. By pooling several 24-hour specimens it was possible, by the biological assay, to detect small amounts of adrenal corticoids in the urine of these infants. The glucocorticoids ranged from 4 to 17 μg/24 hours, the average excretion being 11 μg/24 hours. During the first five days of life no difference could be detected between the full term infant and the premature infant. The glucocorticoid excretion remained at approximately the same level up to the 10th day of life and after that time there appeared to be a gradual increase in the excretion with advancing age in both groups of babies.

In Table I are listed the findings on two newborn babies with atelectasis. These were premature babies born of diabetic mothers. Both babies, during the period of stress, excreted greater amounts of

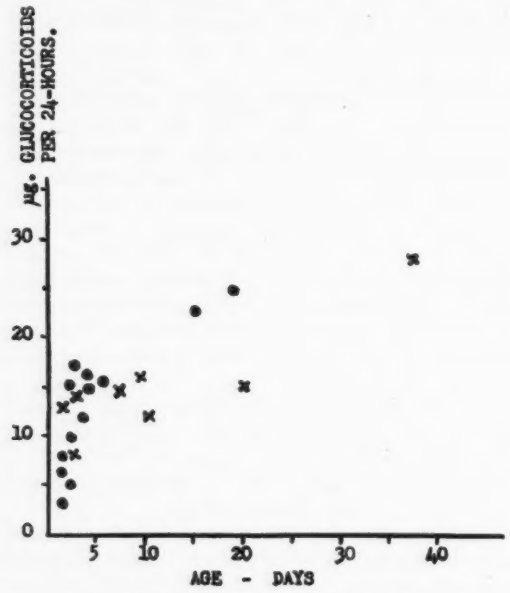

FIG. 1.—Excretion of glucocorticoids in full term and premature babies.
●—Full term
X—Premature

glucocorticoids than the healthy premature babies, 22 µg and 53 µg/24 hours respectively. As the condition of Baby B improved the urinary corticoids returned to lower levels, 13 µg/24 hours. When the assay was repeated at the age of 54 days, 27 µg/24 hours glucocorticoids were found.

TABLE I

Babies	Age days	Glucocorticoids µg/24 hours	Remarks
Baby M	1– 3	22	moderate atelectasis
Baby B	1– 3	53	severe atelectasis
	7– 9	14	lungs clear
	11–13	13	normal
	54–56	27	normal

It was not possible to detect any glucocorticoids in the extracts of the three placentae examined.

DISCUSSION

As glucocorticoids were found to be present in the urine of the newborn infant, it must be concluded that the adrenal gland is capable of elaborating these hormones at birth. The possibility that these substances might be transmitted through the placenta from the maternal circulation seems doubtful as, in many instances, the urine collection was not begun until the third day of life, also it was not possible to detect any activity in the placenta itself. The urine obtained from infants born prematurely (4 to 8 weeks) also contained small amounts of glucocorticoids and no difference between the full term and the premature infant was observed. In both groups of babies the excretion of glucocorticoids slowly increased with age. According to Benner (1940) growth of both medullary and true cortical tissue proceeds fairly constantly in fetal and post-natal life in spite of the rapid involution of the fetal cortex. As there is a gradual increase in the excretion of glucocorticoids at a time when rapid degeneration of the fetal cortex is occurring, these findings do not support the suggestion that the fetal cortex is elaborating glucocorticoids.

Swinyard (1943) in a cytological study of the adrenal gland of the newborn states that the adult cortex is entirely zona glomerulosa, the zona fasciculata only appearing during the second post-natal week. In studies on the rat adrenal, Deane, Shaw and Greep (1948) have correlated the zona glomerulosa with changes in electrolyte metabolism while morphological and histochemical alterations in the zona fasciculata are correlated with functional tests for the glucocorticoids.

In the newborn infant therefore, glucocorticoids are being elaborated by the adrenal gland before the appearance of the zona fasciculata.

That the adrenal gland of the newborn infant is capable of responding to stress in a manner similar to that observed in adults by Venning and Browne (1944), is indicated by the findings in the two premature babies suffering from atelectasis. In Baby B, there was a marked increase in output of glucocorticoids at a time when the damage was most severe. As the lungs cleared, the excretion of these substances returned to levels found in healthy babies of the same age. At 54 days of age, this baby was excreting approximately twice the average amount of glucocorticoids found at birth.

CONCLUSIONS

The newborn baby excretes small amounts of glucocorticoids.

There is no apparent difference between the amount of glucocorticoids excreted by the full term or the premature infant.

The excretion of glucocorticoids gradually increases with age of baby.

This gradual increase in the excretion of glucocorticoids coincides with the rapid involution of the fetal cortex. This fact does not seem to support the suggestion that the fetal cortex of the newborn is elaborating glucocorticoids.

The adrenal gland of the newborn is capable of responding to severe trauma with an increased output of glucocorticoids.

No glucocorticoids could be detected in placental extracts.

ACKNOWLEDGMENT

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DESOXYCORTICOSTERONE ACETATE: STUDIES ON THE REVERSIBILITY OF ITS EFFECT ON BLOOD PRESSURE AND RENAL DAMAGE IN RATS[1]

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IN PREVIOUS studies it has been reported that the administration of desoxycorticosterone acetate (DCA) to rats, when accompanied by a liberal intake of sodium chloride, leads to cardiac and renal enlargement (Selye, 1940; Green, Coleman and McCabe, 1948; Knowlton, Stoerk, Seegal and Loeb, 1946), renal damage (Selye 1943) and to the development of hypertension (Grollman, Harrison and Williams 1940; Selye and Hall, 1943). The alterations produced by this steroid are much intensified in the presence of pre-existing renal damage. With the experimental conditions employed in this laboratory, hypertension following DCA injections has been observed only in rats previously rendered nephritic by a cytotoxic serum (a rabbit anti-rat kidney serum). In these hypertensive animals, a more rapid progression in the course of the pre-existing nephritic process has been found.

The present study was undertaken in an attempt to determine whether the hypertension in rats given anti-kidney serum and DCA was a phenomenon dependent upon its acceleration of the renal damage, or whether it was due to another effect of the steroid. Were the former assumption correct, discontinuation of the steroid injections, once hypertension was established, should have no significant effect upon the blood pressure. On the other hand, were the latter concept correct, a reversal of the hypertensive levels should occur upon withdrawal of DCA.

An additional facet of the problem was studied at the same time. It has been shown that a normal or high intake of sodium chloride is essential for the effects of DCA to become manifest (Knowlton, Loeb, Stoerk and Seegal, 1947). In view of this, it seemed of interest to determine whether rigid sodium chloride restriction might influence the course of development of the cytotoxic nephritis itself without DCA.

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METHODS

Forty-nine male rats of the Long-Evans strain, ranging in age from 68–83 days, at the start of the experimental period, were divided into three groups of 26, 15 and 8 each. The details of the allocation of the animals are shown in Table I. Groups I and II received a modification of the McCollum diet previously described (Smadel, 1936); in this instance, it was adjusted to contain 1.7 per cent sodium chloride. For Group III, sodium chloride was omitted from the diet. Previous analyses of this low sodium diet have revealed a sodium content of 0.89 m.Eq. per 100 gm. of diet. All animals received tap water to drink, and fluid intake was measured daily.

For two weeks prior to the beginning of the study, and at weekly intervals during the experimental period, blood pressure determinations were made, using a modification of the plethysmographic method, as previously reported (Loeb, Knowlton, Stoerk, and Seegal, 1949).

A qualitative albumin determination was made on the urine of each animal prior to the beginning of the study. During the experimental period, quantitative urine albumin determinations (Shevky and Stafford, 1935) were made at intervals on each rat.

On the first day of the experimental period, all animals were injected intravenously with a rabbit anti-rat kidney serum, prepared as described by Smadel (1936). A pool of sera from rabbits #653 and #663 was used, each rat receiving 0.4 cc. on two successive days, i.e., a total of 0.8 cc. per rat.

Beginning the second day of serum administration, daily injections of 2.5 mg. of DCA[2] were initiated in the rats of Group I. The DCA preparation contained 10 mg. per cc. in peanut oil; 0.25 cc. was given at each injection, subcutaneously.

In the seventh experimental week, the nephritic DCA treated rats of Group I were subdivided as follows: one-third were sacrificed (Ia), in one-third, DCA administration was continued for an additional nine weeks (Ib), while in the final third, DCA injections were discontinued (Ic) and the animals observed over a period comparable to those in Ib. As a control for Group Ia, half of the nephritic non-DCA treated animals of Group II were sacrificed in the seventh week (IIa), while observations were extended on the remainder of this group (IIb) for an additional nine-week period. At the end of this time, all rats were sacrificed.

At sacrifice, the animals were first anesthetized with ether, then 3 cc. of blood was withdrawn from each rat under oil and the blood pooled according to group. On this pooled blood, determinations of sodium and potassium (Berry, Chappell and Barnes, 1946), chloride (Wilson and Ball, 1928), total protein (Lowry and Hunter, 1945) and serum urea nitrogen (Gentzkow, 1942), were done. An exception was made in the nephritic, non-DCA injected rats sacrificed at seven weeks (IIa). In this group, individual blood samples were analyzed for serum urea nitrogen, and no other determinations were made. At autopsy, these animals were weighed, and organ weights recorded for heart, kidneys and adrenal glands. Sections of these organs were fixed in Zenker's solution for histological study.

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[2] We wish to thank Dr. Kenneth Thompson of Roche-Organon, Nutley, N. J. for his generosity in providing the DCA.

TABLE I. ALLOCATION OF ANIMALS

Group	No. of rats	Diet	Anti-Kidney serum	DCA	Date of Sacrifice
I. a.	8	1.7% NaCl	0.8 cc.	2.5 mg. daily for 7 weeks	7th week
b.	8	1.7% NaCl	0.8 cc.	2.5 mg. daily for 16 weeks	16th week
c.	10	1.7% NaCl	0.8 cc.	2.5 mg. daily for 7 weeks	16th week
II. a.	7	1.7% NaCl	0.8 cc.	None	7th week
b.	8	1.7% NaCl	0.8 cc.	None	16th week
III.	8	Low NaCl	0.8 cc.	None	16th week

RESULTS

During the experimental period, two rats in Group I and two in Group II died. The blood pressures of these animals were comparable to those of others in their respective groups. They have, however, been excluded since three died of intercurrent pulmonary infections, while the fourth died in uremia with a unilateral hydronephrosis. The remaining 45 animals gained weight and completed the experimental period.

Blood Pressure

The detailed data for all groups are presented in Table II; i.e., the weekly average blood pressure reading of all rats in each group is tabulated and the range of values obtained in individual animals is also included. In addition, a portion of the data is presented in graphic form (Graph I).

From this graph and table, it can be seen that all the nephritic animals receiving DCA (Group I) showed a progressive rise in blood pressure for the first nine weeks, the tension then stabilizing at levels of 200 mm. Hg or over. The rise in blood pressure occurs somewhat more slowly in Group Ib, due to the fact that the more hypertensive animals were purposely selected after seven weeks for sacrifice and for DCA withdrawal, leaving the less severely hypertensive rats for continuance of DCA.

Withdrawal of DCA resulted in a fall in the average blood pressure level although, as can been seen in Table II, Group Ic, individual animals showed persistent severe hypertension (three of the nine animals had tensions greater than 190 mm. Hg at sacrifice). The decline in the average blood pressure did not continue downward to normal levels; however, it approached the levels seen in the nephritic Group II animals, which received *no* DCA. Among these latter rats, a moderate degree of hypertension (around 150 mm. Hg) became evident after the tenth week of the experimental period.

TABLE II. TABLE OF WEEKLY BLOOD PRESSURE READINGS

	Group I						Group II				Group III	
	(a) AKS+NaCl +DCA		(b) AKS+NaCl +DCA		(c) AKS+NaCl +DCA (for 7 weeks)		(a) AKS+NaCl		(b) AKS+NaCl		AKS+Low NaCl	
No. of Animals	8		7		9		7		6		8	
	Aver.	Range	Aver.	Range	Aver.	Range	Aver.	Range	Aver.	Range	Aver.	Range
Weeks 1	119	110 131	120	114 126	120	93 144	125	121 131	123	115 129	119	109 137
0	123	106 136	119	106 134	118	111 127	126	106 143	123	115 132	124	109 142
Experimental 1	124	111 133	120	110 127	122	111 135	125	110 143	111	109 114		
2	130	105 148	128	118 140	122	114 140	126	114 137			119	112 128
3	152	130 181	143	125 151	153	135 171	126	110 150	109	97 120		
4	168	130 187	171	153 196	166	149 175	132	118 153			118	110 127
5	183	172 192	171	153 196	178	160 204	132	120 146	117	108 120		
6	188	177 202	180	153 199	189	171 231	139	125 151	126	121 129	131	116 145
7	196	183 219	188	157 214	206*	194 219	140	126 153	121	113 126		
8			180	158 197	182	155 211			119	108 129	119	106 133
9			209	177 243	169	134 203			123	116 130	148	139 162
10			213	194 243	189	169 226			128	117 152	140	128 161
11			217	210 243	177	148 210			146	123 176	142	133 150
12			217	208 233	163	130 183			149	128 198	143	136 169
13			216	185 239	160	138 200			146	127 186	140	127 160
14			213	193 233					150	133 173	152	137 165
15			220	208 235	168	130 227			153	126 183	153	137 180
16					176	150 231						

* DCA discontinued after this week.

Finally, it is of interest that in the two groups of nephritic rats given *no* DCA, the blood pressure level was not influenced by rigid sodium chloride restriction (cf. Groups II and III, Table II).

Fluid Intake

The average daily ingestion of water per rat per week is presented in Graph II. The DCA treated animals showed an increased intake compared to both non-DCA injected groups, in the early weeks of the experiment. Discontinuance of the steroid (Ic) was accompanied by a

decrease in water intake. This finding is in harmony with earlier reports in dogs treated with DCA (Ragan, Ferrebee, Phyfe, Atchley and Loeb, 1940). Toward the end of the four-month experimental period, there was an increased intake in all groups, although this was minimal in the nephritic rats of Group III on sodium chloride restriction. The animals in which DCA was continued throughout the study, Ib, ingested the largest amount of fluid, as might be expected.

Albuminuria

No abnormal urinary albumin, as determined by the heat and acetic acid method was observed prior to the injection of anti-kidney serum. Following these injections, all animals developed significant albuminuria (over 5 gm. per liter) (Table III). Because of the marked variation from animal to animal, little importance can be ascribed to the averages presented.

Chemical Determinations

These are summarized in Table IV. The serum sodium determination on pooled sera from DCA treated rats sacrificed after 16 weeks, Ib, was elevated, with an associated slight reduction in serum potassium. This is a characteristic effect of DCA (Ferrebee, Parker, Carnes, Gerity, Atchley and Loeb, 1941). In DCA treated animals sacrificed after seven weeks, no alteration in sodium or potassium was observed. Serum chlorides and serum proteins were essentially similar in all groups. In both groups of animals sacrificed at seven weeks, i.e., the DCA treated rats of Ia and the non-DCA injected animals of IIa, serum urea nitrogen values were just above normal limits. In the animals receiving DCA daily for 16 weeks (Ib), the serum urea nitrogen value was again just above the normal limits, while in the rats in which DCA had been discontinued (Ic), as well as in both groups of animals without DCA (IIb, III), significantly higher levels were observed. Whether or not the difference in serum urea nitrogen levels might be ascribed to differences in filtration rate was not established in this study.

Autopsy Findings

The autopsy findings are summarized in Table III.

Body Weight

The body weights were comparable in the various groups, although, as might be expected, those animals sacrificed at seven weeks weighed slightly less than those sacrificed nine weeks later.

Kidneys

The anti-kidney serum employed in these experiments produced renal enlargement and histological evidence of nephritis in all rats.

The kidney damage involved all portions of the renal tissue, i.e., glomeruli, tubules and interstitial elements. A comparison of the nephritic non-DCA injected rats killed at seven (IIa) and at sixteen weeks (IIb), reveals that with the passage of time, definite progression of the glomerular lesions occurs, although the tubular and inter-

TABLE III. TABLE OF FINAL BLOOD PRESSURE, BODY AND ORGAN WEIGHTS, HISTOLOGICAL FINDINGS, ALBUMINURIA

	Group I			Group II		Group III
	AKS[1]+DCA[2]+1.7% NaCl diet			AKS[1]+1.7% NaCl diet		AKS[1]+Low NaCl
Date of Sacrifice	(a) 7 weeks	(b) 16 weeks	(c) 16 weeks (DCA stopped after 7 weeks)	(a) 7 weeks	(b) 16 weeks	16 weeks
Number of Rats	8	7	9	7	6	8
Final Blood Pressure Average Range	196 (183–219)	220 (208–235)	176 (150–231)	140 (126–153)	153 (126–183)	153 (137–180)
Body Weight Gm. Average Range	283 (220–340)	290 (275–315)	303 (240–340)	277 (245–300)	281 (236–322)	290 (230–330)
Kidney Weight Gm. Average Range	3.78 (3.10–4.46)	3.81 (3.23–4.24)	3.71 (2.98–5.87)	3.90 (2.56–3.94)	3.97 (3.26–4.78)	3.35 (2.59–4.06)
Animals with Nephritis	8/8	7/7	9/9	7/7	6/6	8/8
Urine Albumin Gm/L 6th week	54 (13–92)	38 (11–97)	23 (8–40)	27[3] (22–43)	70 (28–105)	59 (7–135)
15th week	—	18 (10–30)	26 (12–40)	—	41 (21–61)	44 (12–88)
Heart Weight Gm. Average Range	1.22 (1.01–1.49)	1.46 (1.23–1.59)	1.23 (.87–1.48)	.83 (.81–1.00)	1.01 (.93–1.10)	.94 (.81–1.13)
Adrenal Weight mg. Average Range	42 (32–49)	42 (39–51)	43 (39–55)	44 (36–60)	47 (41–50)	47 (39–56)
No. Rats Showing DCA Effect in Adrenals	7/7[4]	6/6[4]	3/8[4]	1/7	3/5[4]	0/7[4]

[1] AKS = Rabbit, anti-rat kidney serum.
[2] DCA = Desoxycorticosterone acetate in oil, 2.5 mg. daily.
[3] Average of 3 animals.
[4] One pair of adrenals lost in preparation.

stitial damage was no more severe in the group sacrificed at the later date. Clinically, evidence of renal damage was more marked in the 16-week animals, i.e., blood pressure elevation and retention of urea nitrogen.

In earlier studies, in which less potent cytotoxic sera were employed, DCA definitely enhanced both renal hypertrophy and renal damage. In the present experiments, no augmentation of renal size was observed during steroid administration. Histologically, there was

no striking increase in renal damage which could be ascribed to DCA. It was again noted that the rats sacrificed at four months (Ib) had more glomerular damage than the similarly treated group sacrificed nine weeks earlier (Ia). In view of the fact that no augmentation of renal damage was observed in the DCA treated groups, it is not surprising that no striking differences were noted between these rats and the group in which DCA had been discontinued after the initial seven weeks of the sixteen week experimental period (Ic).

Finally, a comparison of the two nephritic groups which did not receive any steroid injections, reveals that the kidneys of the animals

TABLE IV. TABLE OF CHEMICAL DETERMINATIONS ON POOLED SERA

Group	Serum Na m Eq./L	Serum K m Eq./L	Serum Cl m Eq./L	Serum protein gm/100 cc	Serum urea nitrogen mg./100 cc.	Date of sacrifice
I. a) DCA+AKS+NaCl	143.4	5.0	94.4	6.0	25	7th week
b) DCA+AKS+NaCl	151.6	3.9	95.0	6.1	24	16th week
c) DCA for 7 wks. +AKS+NaCl	145.0	5.6	100.4	5.8	39	16th week
II. a) AKS+NaCl	—	—	—	—	23	7th week
b) AKS+NaCl	143.2	4.9	97.4	5.7	43	16th week
III. AKS+Low NaCl	146.8	4.8	97.8	6.1	44	16th week

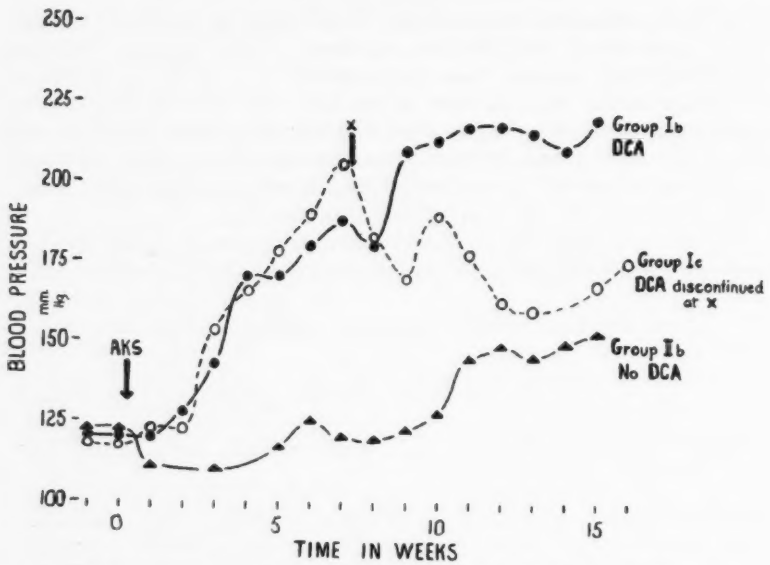
on 1.7% sodium chloride diet (IIb) were slightly heavier than those of the rats on sodium restriction (III). Histologically, however, the evidences of renal damage were not significantly different.

Heart Weights

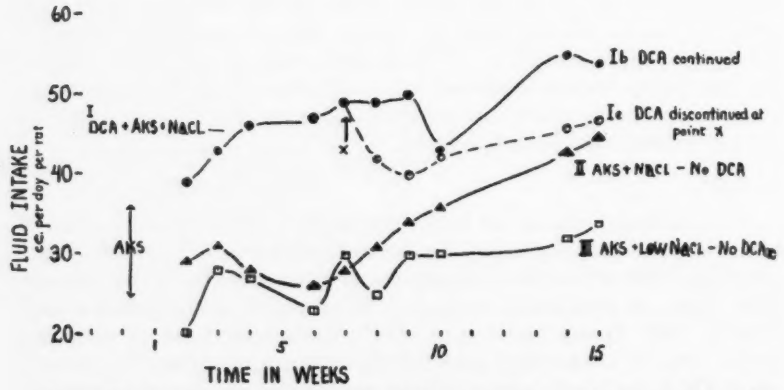
DCA, as previously observed, produced an increase in cardiac size. In those animals (Ic) to which DCA had been given initially but discontinued nine weeks before sacrifice, the heart weights were less than those of animals receiving DCA for the full 16 weeks of the experiment; that is the cardiac hypertrophy did not continue to progress after discontinuation of the steroid. However, it can not be stated that the hypertrophy regressed after withdrawal of DCA, since the heart weights were no smaller than those of rats (Ia) killed at the time DCA was discontinued in Group Ic.

Adrenals

No significant alteration in the size of the adrenal glands occurred in any group. Histologically, the adrenal glands of all nephritic rats receiving DCA at the time of sacrifice showed atrophy of the subcapsular zone as previously reported (Carnes, Ragan, Ferebee and O'Neill, 1941; Greep and Deane, 1947; Knowlton, Loeb, Stoerk and Seegal, 1947). Of the eight adrenal glands examined from the rats in which DCA had been discontinued, only three showed this change, indicating the reversibility of this atrophy. It is noteworthy that in the nephritic group receiving sodium chloride, but *no* DCA (II), the



GRAPH I.—Average weekly blood pressure readings Groups Ib, Ic, IIb.
AKS: Rabbit anti-rat kidney serum given to all animals.
Group Ib: AKS+1.7% NaCl diet+DCA 2.5 mg. daily for 16 weeks.
Ic: AKS+1.7% NaCl diet+DCA 2.5 mg. daily for first 7 weeks.
IIb: AKS+1.7% NaCl diet. No DCA.



GRAPH II.—Average daily fluid intake of all groups expressed in cc. per rat per day, charted by week.
AKS: Rabbit anti-rat kidney serum given to all animals.

adrenal gland of one animal sacrificed at seven weeks and three animals sacrificed at sixteen weeks, showed a similar subcapsular atrophy, the so-called "DCA" change. None of the adrenals of animals from the nephritic group on sodium restriction (III) showed subcapcular atrophy. Indeed, in six of the seven pairs of glands examined, there was distinct hyperplasia of the subcapsular zone.

DISCUSSION

In previous studies in this laboratory (Knowlton, Stoerk, Seegal and Loeb, 1946; Knowlton, Loeb, Stoerk and Seegal, 1947; Loeb, Knowlton, Stoerk and Seegal, 1949), the cytotoxic sera employed have induced minimal evidence of renal damage and no elevation of blood pressure within the periods of observation, which extended up to eight weeks. In the present experiments, the anti-kidney serum proved of considerably greater potency. A severe nephritis was found in the greater majority of rats sacrificed at seven weeks (IIa). Furthermore, in animals observed over a four-month period (IIb), there occurred in addition to histological renal damage, an elevation in serum urea nitrogen terminally and in the majority of the rats, moderate hypertension, while an occasional animal had blood pressures in excess of 175 mm. Hg.

Against this background of extensive renal damage induced by the cytotoxic serum, the accentuating effects of DCA are less evident than those observed previously. However, the increased elevation of blood pressure which ensued when DCA was administered remains impressive. With DCA, the average blood pressure at the time of sacrifice was in excess of 200 mm. Hg, while the nephritic animals without DCA averaged more than 50 mm. Hg lower (Graph I). Bearing in mind that the histological evidence of renal damage was the same in the two groups, one must conclude that the steroid produces an effect on blood pressure not entirely dependent upon demonstrable alterations in renal histology. A further point in favor of this argument is the fact that the hypertensive effect of the steroid is in part reversible upon withdrawal. It is wholly possible that DCA produces its hypertensive effect by means of an alteration in renal blood flow or other functional changes without demonstrable deviations in structure. Why the urea nitrogen in animals with long continued administration of DCA is significantly lower than in all other groups is not apparent at this time. As all animals were on a similar diet and gained similar amounts of weight, the lowered serum urea nitrogen cannot be ascribed to a lowered protein intake in the DCA treated group. Since the serum protein values were similar in all groups, hemodilution can not explain the phenomenon. Furthermore, the increased fluid intake of these DCA treated animals does not seem sufficient to account for the lower urea nitrogen. The low values for serum urea nitrogen in the animals receiving DCA cannot be harmonized

with the lowered blood flow and filtration rate in DCA treated rats reported by Friedman, Polley and Friedman (1948).

The changes in the subcapsular zone of the adrenal gland, seen consistently in DCA treated animals, is apparently not specific for this steroid. In a previous study it was noted that these atrophic changes did not occur following DCA if the sodium intake of the rats was restricted (Knowlton, Loeb, Stoerk and Seegal, 1947). In the present experiments, similar atrophic changes were observed in a number of nephritic animals which had never received DCA, but which were on a relatively high sodium chloride intake (1.7%). It would appear that this is a further instance in which such changes can result from sodium chloride alone, and that DCA acts to intensify the effect of the salt. Furthermore, it is of interest that in the presence of sodium restriction, without DCA, the reverse of atrophy was observed, i.e., actual hyperplasia of this zone of the cortex.

Finally, as to the effects of sodium restriction upon the course of the cytotoxic nephritis, the development of hypertension, urea nitrogen retention and renal damage were not materially altered by rigid reduction of sodium chloride, when a highly potent nephrotoxic serum was employed, as in this study.

CONCLUSIONS

A severe nephritis was produced in rats with the injection of a potent rabbit anti-rat kidney serum.

Hypertension of a moderate degree developed in these nephritic rats independently of variation in sodium chloride intake and without the administration of DCA.

The administration of DCA accelerated the appearance of, and greatly augmented, the hypertension in such nephritic rats.

Withdrawal of DCA, after severe hypertension had been established, resulted in a decrease in blood pressure.

The intensity of the nephritis in this study was not augmented by prolonged administration of DCA, perhaps because of the unusual severity of the process resulting from anti-kidney serum alone.

Subcapsular atrophy of the adrenal cortex, regularly encountered following DCA plus sodium chloride, was observed in some nephritic animals receiving sodium chloride but no DCA. No nephritic animal on rigid sodium restriction showed this change.

Adrenal cortical changes suggestive of hyperplasia of the subcapsular zone were seen in nephritic animals on salt restriction.

ACKNOWLEDGMENTS

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NEW BOOK

THE THYROID AND ITS DISEASES. 2nd Edition 1948. J. H. Means. J. B. Lippincott Co., Publishers. Philadelphia. vii+571 pp. $12.00.

The first edition of Doctor Means' *Thyroid and Its Diseases* was published in 1937, and the second edition is thoroughly revised to present important advances made in the intervening years.

The plan of presentation remains much the same as in the earlier edition; first anatomical considerations of the thyroid gland are given, followed by a discussion of the nature of its hormone. These chapters cover their subjects in detail, are well documented, and provide instructive reading. The chapter which follows bears on the relations of the thyroid gland to other endocrine glands and is introduced by statements on the similarity of the endocrine system to the nervous system in integrative function. There is a declaration that an attempt will be made to distinguish sharply between well-established facts and uncertain evidence. This attempt is usually successful, but, unfortunately, not regularly so. For example, experimental evidence on the mode of action of thiocyanate and thiouracil on iodine metabolism by the thyroid gland is presented schematically; thiocyanate is shown as imposing a block to the completion of thyroid hormone distal to the uptake of iodine and thiouracil, a block proximal to iodine uptake. Numerous publications have shown that this presentation is incorrect, and indeed, the mode of action of these two substances is such that if "thiocyanate" and "thiouracil" were exchanged in the schema the true situation might be quite accurately portrayed. That this is true is partly recognized in the text although no notice of the contradiction is taken.

The pathology of the thyroid gland in this edition is presented by R. W. Rawson beginning with a review of normal thyroid structure and its alteration by increased need for thyroid hormone and by antithyroid drugs. The section on adenomas follows the classification of Wegelin and that on cancer, of Warren. It is noteworthy that substances such as thiocyanate are said to produce goiters which simulate adenocarcinoma and that Riedel's struma may be mistaken clinically and by microscopic examination for cancer, emphasizing the difficulties of interpretation in this field.

The remainder of the book is concerned with clinical considerations. Symptomatology, methods of examination in thyroid diseases, myxedema, cretinism, and hyperthyroidism are presented in order. Chapters on tumors (by R. W. Rawson), thyroiditis, anomalies, surgery of the thyroid gland (the chapter on Surgery is written by O. Cope), on non-thyroidal diseases treated with thyroid hormone, and on "Facts and Fancy in Matters Thyroid" tend to follow the previous edition, with appropriate revision.

The clinical sections on myxedema and cretinism are delightfully written and stem from a wealth of experience with these disorders. Most clinicians should profit greatly from reading them and will find, in addition to the author's accurate descriptions, well-chosen selections from earlier writings, one from Ord being outstanding to the reviewer. The differential diagnosis

of so-called pituitary myxedema from true myxedema, however, seems not at all in keeping with the otherwise high quality of the section. At least, the reviewer doubts strongly that follicle-stimulating hormone, 17-ketosteroid, and sodium and chloride determinations will often aid in the distinction between them, and in regard to treatment the statement that "an anterior pituitary preparation like chorionic gonadotrophin" may be used in treating pituitary myxedema is distressing on two scores.

As in the first edition a considerable portion of the book is devoted to hyperthyroidism. The classification has been altered; in the first edition the designations of classic exophthalmic goiter or Graves' disease and of toxic nodular goiter or Plummer's disease were used for the clinical separation of toxic goiter, the author, however, expressing doubt as to the validity of the distinctions. The present edition departs from this practice in favor of the term Graves' disease, Plummer's being relegated to another section. Graves' disease, in this edition, is subdivided into the thyrotoxic group, occurring with or without "ophthalmopathy," and into a so-called "hyperophthalmopathic" group, attended by hyperthyroidism, euthyroidism, or hypothyroidism. The incidence of the "hyperophthalmopathic" phase is not made clear but the emphasis on it seems dictated rather more by the author's interests than by the frequency of its occurrence. It is stated without documentation that treatment with irradiation by Roentgen ray or radioactive iodine in cases with severe eye disorders and hyperthyroidism is preferable to thyroidectomy or antithyroid drugs. The objection raised to antithyroid drugs is stated as follows: "They produce what has been called a medical thyroidectomy and are to be avoided for the same reasons as surgical thyroidectomy." Doctor Means avoided a good deal of confusion in this argument by overlooking Dr. Cope's statement in the same book, "The medical therapy of hyperthyroidism with radioactive iodine is essentially a thyroidectomy."

The account of the introduction of antithyroid drugs is sufficiently unusual to warrant quoting: "In our own clinic, interest in the possibility of treating thyrotoxicosis by antithyroid drugs began October 16, 1941. On that day we presented at medical grand rounds a patient who had developed myxedema and hyperplastic goiter while taking potassium thiocyanate as treatment for hypertension. Because of the hypothyroidism that developed as a result of the therapy with this drug, Dr. Earle M. Chapman suggested that potassium thiocyanate might be used in treating hyperthyroidism. Four thyrotoxic patients were, accordingly, treated with this agent. One made a good response, three did not." This would appear an interest heightened in retrospect, for Doctor Means in writing a Medical Progress report for the *New England Journal of Medicine* in October, 1942, made no mention of this interest in antithyroid drugs.

In 1942 Doctor Means stated "the prophecy that surgery will be used less and less in this field (Graves' disease) as the endocrinology becomes better understood," and he noted "Of the treatment of Graves' disease in general it is interesting that in the Thyroid Clinic of the Massachusetts General Hospital we have come full circle in a quarter century. In the years 1919–1922, half the cases were treated by non-surgical methods. In 1932–1935 surgery was employed in 95 per cent. In 1942, half surgical, half non-surgical procedures are being employed." There is in this edition a very sporting

discussion of the relative merits of the several methods now available for treating Graves' disease; and it is interesting to note that in spite of prophecy the circling continues. By 1948 the Thyroid Clinic, having hurdled the radioactive iodine and antithyroid drug obstacles, was running with an easy surgical swing along the 1932–1935 backstretch, a feat clearly establishing its right to lead the field.

Doctor Means has a handsome flair for turning a phrase. He has renamed the old chapter on "Simple Goiter," remarking "Now with the acquisition of new knowledge regarding the action of iodine and the action of goitrogens the matter has become very far from simple. It is almost as if it were the doctors who were simple—not the goiters." The bedclothing difficulties which occur with couples, one of whom has thyroid disease, is referred to as "this thermal variety of connubial maladjustment." "Struma cibaria" and "struma medicamentosa" for goiters due to foods and to drugs seem useful innovations. In regard to "struma cibaria," however, it seems doubtful that even a starving monk could stomach appreciable amounts of rape seed with his cabbage despite the statement to this effect in the text. That "thyropriviac," "to thiouracilize," and "thiouracilization" will prove sufficiently useful to warrant retention is open to question.

Following the main sections of the book there is a chapter on "Thyroid Administration in Diseases of Other Than Thyroid Origin," the use of the hormone for its nonspecific drug action, as it is stated. Doctor Means does not hold to the point of view that the exhibition of small doses of thyroid hormone to euthyroid patients suppresses endogenous hormone production and believes it proved by practice that advantage may be taken of the calorigenic or other action of the hormone and makes an emphatic distinction between the physiologic action in myxedema and the pharmacologic action in conditions not etiologically related to the thyroid. Neither the distinction nor the practical proof is altogether convincing; in regard to the latter the discussion of sterility and habitual abortion provides a case in point. Physicians are enjoined to prescribe thyroid for all cases of sterility not due to local disease regardless of substandard or standard basal metabolism. This recommendation is based on the impressions of many clinicians, but no data are provided which establish the matter; there is no mention of the great difficulties in evaluating results of treatment in sterility and abortion. Undoubtedly physicians are often called upon to do something, that is, to conduct clinical experiments. Unfortunately, reasonable experimental conditions are seldom met; proper selection of cases, recording of data and comparison with control material usually are not made. The final result is that it is often not clear despite years of trial whether patients have benefited from a treatment, or have withstood it.

This volume is clearly outstanding in the thyroid field; the late Dr. Joll's book and several others are now well out-of-date, the late Dr. Hertzler's suffered from its intensely personal nature, and Dr. Crile's new volume is restricted to practical aspects of thyroid disease. It is comprehensive in its scope, and is written with perspective and in a charming, leisurely way. Its judgments are almost always well tempered and balanced, seldom dogmatic. Although this review is critical in tone, it would be ill indeed not to declare the defects minor, conspicuous only by contrast with the general merit of this excellent book.

ANNOUNCEMENT OF THE 1950 MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirty-Second Annual Meeting of The Association for the Study of Internal Secretions will be held at the Sir Francis Drake Hotel, Friday and Saturday, June 23 and 24, 1950, in San Francisco, California.

The Committee on Local Arrangements is comprised of Dr. Hans Lisser, Chairman and Doctors Leslie L. Bennett, Roberto F. Escamilla, Minnie B. Goldberg, Gilbert S. Gordan, Laurance W. Kinsell.

Hotel accommodations will be difficult to secure on short notice; therefore, members are urged to make their reservations at once. All requests must be addressed to: Dr. William Howard Rustad, American Medical Association (Hotel Committee), Room 200, Civic Auditorium, San Francisco, California.

The scientific sessions will be held in the Empire Room of the Sir Francis Drake, and registration will be on the same floor. The annual dinner will be held in the Empire Room on Friday, June 23 at 7:30 P.M., preceded by cocktails at 6:30 P.M.

Those wishing to present papers, which will be limited to ten minutes, should send title and four copies of an abstract of not more than 200 words, to Edward A. Doisy, M.D., St. Louis University School of Medicine, 1402 South Grand Avenue, St. Louis, Missouri, not later than March 1, 1950. *It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing in the program and journals of the Association.* Names of non-members who are co-authors must be followed by the words "by invitation," and the principal degree of each author.

Nominations for the Ayerst, McKenna and Harrison Fellowship, the Schering Fellowship in Endocrinology, and the Squibb and Ciba Awards should be made on special application forms which may be obtained from the Secretary-Treasurer, Henry H. Turner, M.D., 1200 North Walker Street, Oklahoma City 3, Oklahoma, and filed with the Secretary not later than March 15, 1950.

Announcing

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The faculty will consist of prominent researchers and clinicians in the field of endocrinology and metabolic disorders, gathered from the United States and Canada.

The course will be a practical one of interest and value to the specialist and those in general practice. The program will consist of lectures, clinics and demonstrations. Ample time will be given to questions and answers at the end of each session, and registrants are encouraged to contact members of the faculty for individual discussions.

The Roney Plaza, one of Miami Beach's most delightful hotels, offers special convention rates to members of this assembly. This is an unusual opportunity for you and your family to enjoy a pleasant vacation and for you to participate in a highly instructive program of the latest advances in endocrinology and metabolism.

A fee of $75 will be charged for the entire course and the attendance will be limited to 100. REGISTRATION WILL BE IN THE ORDER OF CHECKS RECEIVED AND WILL CLOSE ON MARCH 3, 1950. Should there be an insufficient number of applicants to fill the course, the registration fee will be refunded immediately in its full amount.

Application for approval of this course has been made to the Veterans Administration. Veterans should make formal application to their local agencies on the appropriate form (1905e or 1950) as furnished by the V.A.

Please forward application on your letterhead together with check payable to The Association for the Study of Internal Secretions, to Henry H. Turner, M.D., Secretary-Treasurer, 1200 North Walker Street, Oklahoma City 3, Oklahoma, before March 3, 1950. Further information and program will be furnished upon request.

Hotel reservations should be made directly with the Roney Plaza Hotel, Miami Beach, Florida, and the hotel advised that you are attending this Postgraduate Assembly.